A clinicopathological and molecular categorisation of serrated colorectal polyps

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BSc MBBS FRCPA

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School of Medicine
Abstract
Approximately 25% of colorectal carcinomas arise via the serrated neoplasia pathway. These cancers develop in serrated type polyps. Removal of these precursor polyps before the development of malignancy offers a unique opportunity to interrupt neoplastic progression and is the rationale for colonoscopic surveillance.

Serrated colorectal polyps fall into three major subtypes; namely hyperplastic polyps, sessile serrated adenomas and traditional serrated adenomas. Hyperplastic polyps are the most numerous, but have limited, if any malignant potential. Sessile serrated adenomas give rise to the majority of serrated neoplasia pathway malignancies. Although rare, traditional serrated adenomas may be more likely to progress, thus requiring closer surveillance. While both are amenable to colonoscopic removal they present unique challenges when compared to the polyps of the “traditional” colorectal cancer pathway. In particular, sessile serrated adenomas are sessile (hence the name) and difficult to detect by colonoscopy, they are frequently misdiagnosed/under-diagnosed by pathologists, resulting in inadequate surveillance and their molecular biology is incompletely understood. Thus the aims of this PhD were to address issues relating to the diagnostic criteria of serrated colorectal polyps and to better define the clinicopathological and molecular features of these lesions.

To this end several study sets were established. Firstly, a cohort to address issues surrounding the diagnosis of the sessile serrated adenoma was collected. Although recognised as a distinct entity for over ten years, uniform diagnostic criteria for the sessile serrated adenoma have not yet been established. In particular the distinction of this polyp from a closely related entity, the microvesicular hyperplastic polyp, has not been adequately addressed. Thus a central review of a consecutive series of 6340 colorectal polyps was undertaken. During the review, the diagnostic criteria of both the WHO and an expert panel convened by the American College of Gastroenterologists were applied to the diagnoses of all microvesicular hyperplastic polyps and sessile serrated adenomas. A comparison of the clinicopathological features of the patients in these separate groups was then performed. This demonstrated a distinct shift in the clinicopathological features at the diagnostic threshold set by the expert panel but not at the threshold set by the WHO. Thus these findings supported the diagnostic criteria of the expert panel to differentiate microvesicular hyperplastic polyps from sessile serrated adenomas. Furthermore, 14.7% of all colorectal polyps met the expert panel diagnostic criteria for a sessile serrated adenoma.
The second study set comprised 200 rigorously diagnosed traditional serrated adenomas. A detailed clinicopathological and molecular assessment was performed on all cases. It was found that traditional serrated adenomas segregated into two key subtypes based on their **BRAF** or **KRAS** mutation status. This important molecular distinction underscored a substantial difference in both the clinicopathological and molecular features of these polyps. In particular **BRAF** mutated traditional serrated adenomas arose from pre-existing sessile serrated adenomas, were more likely to be located in the proximal colon, were more likely to have a sessile growth pattern, were more likely to demonstrate the CpG island methylator phenotype and were more likely to silence **CDKN2A** as they progressed to carcinoma. Importantly traditional serrated adenomas of all types retained mismatch repair enzyme function, meaning that the **BRAF** mutated subtype is an important precursor of the very aggressive **BRAF** mutated, microsatellite stable subtype of colorectal carcinoma.

The third study set comprised a group of tubulovillous adenomas with serrated architectural features. A detailed clinicopathological and molecular assessment of these polyps was performed and then compared to control sets of traditional serrated adenomas and conventional tubulovillous adenomas. It was found that the serrated tubulovillous adenomas could be reliably diagnosed and had features distinct from both of the control cohorts. Furthermore, they had very high rates of **KRAS** mutation and thus appear to be an important precursor of the **KRAS** mutated subtype of colorectal carcinoma.

The final study set comprised 137 sessile serrated adenomas with dysplasia and or carcinoma. These polyps were subjected to a detailed clinicopathological and molecular assessment, with a particular emphasis on the distinction between mismatch repair deficient and mismatch repair proficient cases. This dichotomy underscores the microsatellite instability status of the polyps and has important implications for the prognosis of the resultant carcinomas. It was found that mismatch repair proficient cases had distinct features. In particular they arose more often in males, at a younger age and more often in the distal colon than mismatch repair deficient cases. Furthermore, they were less likely to show the CpG island methylator phenotype and were more likely to harbour a **TP53** mutation.

In summary, this thesis has provided evidence to direct the diagnosis of the sessile serrated adenoma, has identified key molecular subtypes of both sessile serrated adenomas and traditional serrated adenomas and has better elucidated the pathways by...
which these polyps progress to carcinoma. Finally a novel polyp subtype, namely the serrated tubulovillous adenoma has been proposed that appears to have distinctive histological and molecular features. This improved understanding of serrated colorectal polyps will contribute to better pathological diagnosis and to a more scientific basis for colonoscopic surveillance protocols.
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ANZSRC code: 111203, Cancer Genetics, 50%

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FoR code: 1112, Oncology and Carcinogenesis (100%)
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<td>CpG island methylator phenotype</td>
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<td>ECF</td>
<td>Ectopic crypt formation</td>
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<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
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<td>FFPE</td>
<td>Formalin fixed paraffin embedded</td>
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<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
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<td>MMR</td>
<td>Mismatch repair</td>
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<td>MMRP</td>
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<td>MPHP</td>
<td>Mucin poor hyperplastic polyp</td>
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<td>MSI</td>
<td>Microsatellite instability</td>
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<td>Microsatellite stable</td>
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<td>PI3K</td>
<td>Phosphatidylinositol-3-kinase</td>
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<td>PMR</td>
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<td>5FU</td>
<td>5-fluoruracil</td>
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Chapter 1: The serrated pathway to colorectal carcinoma: Current concepts and challenges

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Key Words

Histopathology, Large Intestine, Colorectal Neoplasms, Colonic Polyps, Serrated Neoplasia Pathway

Abbreviations
CIMP, CpG island methylation phenotype; CIMP-H, high level CIMP; CIMP-L, low level CIMP; CpG, cytosine residue followed by guanine; CRC, colorectal cancer; ECF, ectopic crypt foci; EGFR, epidermal growth factor receptor; GCHP, goblet cell hyperplastic polyp; HP, hyperplastic polyp; MSI, microsatellite instability; MSI-H, MSI high; MSI-L, MSI low, MSS, microsatellite stable; MPHP, mucin-poor hyperplastic polyp; MVHP, microvesicular hyperplastic polyp; PI3K, phosphatidylinositol 3-kinase; SPS, serrated polyposis syndrome; SSA, serrated adenoma/polyp; SSAD, sessile serrated adenoma/polyp with dysplasia; sTVA, tubulovillous adenoma with serrated features; TSA, traditional serrated adenoma; TVA, tubulovillous adenoma; 5FU, 5-fluorouracil.
Abstract

Approximately 30% of colorectal carcinomas develop via a serrated neoplasia pathway, named for the pattern of crypts in the precursor polyps. Molecular abnormalities consistently involve methylation of CpG islands (CIMP) of low (CIMP-L) or high degree (CIMP-H) and activating mutations of the mitogen activated protein kinase pathway components \textit{BRAF} or \textit{KRAS}. Microsatellite instability (MSI) of high level (MSI-H) is often present, allowing for a molecular classification of serrated pathway carcinoma as; 1) \textit{BRAF} mutant, CIMP-H with either a) MSI-H or b) microsatellite stable (MSS); and 2) \textit{KRAS} mutant, CIMP-L, MSS.

Precursor polyps include sessile serrated adenoma (SSA), characterised by proximal location, crypt architectural disturbance and \textit{BRAF} mutation. Microvesicular hyperplasic polyp (MVHP) likely precedes the development of SSA and borderline lesions between MVHP and SSA occur. Cytological dysplasia in SSA portends advanced genetic abnormality and high risk of progression to carcinoma. The traditional serrated adenoma has a predilection for the left colon, tubulovillous architecture, eosinophilic cytoplasm and frequent \textit{KRAS} mutation.

Serrated morphology carcinoma is a new WHO subtype with well-differentiated, mucinous or trabecular patterns. They have frequent KRAS or BRAF mutations and poor prognosis.

This review provides an insight into the histology and molecular mechanisms driving these serrated pathway lesions.
Introduction

Most colorectal carcinomas (CRCs) are presumed to arise in pre-malignant polyps that are amenable to endoscopic resection, yet CRC remains the second leading cause of cancer death in the developed world. At least two distinct molecular pathways underlie most CRCs. Around 70% arise via the well-characterised chromosomal instability pathway\(^1\textsuperscript{-3}\) on which most screening and treatment decisions are based. Over the last two decades, many of the molecular mechanisms of a ‘serrated neoplasia pathway’ accounting for about 30% of CRCs have been determined.\(^1\textsuperscript{,}2\) This has been paralleled by improved endoscopic and pathological recognition of serrated pathway cancers and polyps.

This review will outline our understanding of the molecular basis of the serrated neoplasia pathway and discuss gaps in the current model. Pathological features of the polyps and cancers arising from this pathway will be presented and areas of contention highlighted.

Unraveling a new molecular model of colorectal carcinoma

A series of parallel scientific and medical advances led to the identification of the serrated neoplasia pathway.

SERRATED POLYPOSIS SYNDROME

The first suggestion of a cancer pathway alternate to that of chromosomal instability came from the study of patients with hyperplastic polyposis syndrome (now SPS). When SPS was first described in 1980, HPs, then the only recognised serrated polyps, were considered to be benign, non-neoplastic lesions.\(^4\) Early reports of an association between SPS and CRC received little attention\(^5\textsuperscript{-}9\) and it was not until 1996 that Torlakovic and Snover demonstrated histological differences between the polyps in SPS and sporadic HPs\(^10\). Others reported atypical features in sporadic HPs, ‘mixed polyps’ with features of both HP and conventional adenoma\(^7\) and occasional CRCs arising in HPs.\(^9\)

LYNCH SYNDROME

Lynch syndrome (formerly Hereditary Non-Polyposis Colorectal Cancer Syndrome), an autosomal dominant condition characterised by a high risk of cancer in multiple organs,
particularly the large bowel, results from a germ-line mutation in a DNA mismatch repair gene.\textsuperscript{11} Inactivation of the remaining normal allele results in loss of mismatch repair function and accumulation of mutations in repeated mono, di or tri-nucleotide DNA sequences (termed microsatellites). Most microsatellites are within non-coding DNA but some genes (e.g. TGF\textbeta\textsubscript{RII} and IGFIIR) harbour microsatellites and are particularly prone to mutation in Lynch syndrome.\textsuperscript{12,13} This propensity is termed microsatellite instability (MSI).

MSI\textsuperscript{14-16} and the first germ-line mutation in a mismatch repair gene (MSH2)\textsuperscript{17,18} were identified in 1993, and mutations in other key mismatch repair genes the following year.\textsuperscript{19-21} Consensus as to the most appropriate markers to define MSI was achieved in 1997: at least two of five target loci showing evidence of mismatch repair deficiency was designated MSI high (MSI-H), a single affected locus MSI low (MSI-L) and no affected locus microsatellite stable (MSS).\textsuperscript{22} Overall, about 15\% of CRCs were MSI-H but a germ-line defect in a mismatch repair gene was identified in only 2-3\%. The cause of the other 12-13\% remained unknown.\textsuperscript{23}

DNA METHYLATION

DNA methylation is a physiologic process with a wide range of functions, including genomic imprinting, timing of DNA replication and regulating chromatin structure and gene transcription.\textsuperscript{24,25} In humans most methylation occurs via the activity of DNA methyltransferase, with cytosine residues followed by guanine (CpGs) being particularly prone to methylation. Methylated cytosine is at high risk of deamination to thymine, as such, CpG dinucleotides are uncommon in most of the genome, occurring with a frequency of 20-25\% of that expected by chance. However, interspersed among the CpG depleted DNA are CpG rich regions referred to as CpG islands occurring almost exclusively in promoter regions of genes predominantly coding for housekeeping proteins involved in cell metabolism and cell structure. Methylation of CpG dinucleotides in these islands often results in gene silencing.\textsuperscript{24-26} As many tumour suppressor genes (including \textit{p16} and \textit{MLH1}) harbour CpG islands in their promoter regions, CpG island methylation is a potential mechanism of carcinogenesis.\textsuperscript{27-29}

In 1999, Toyota \textit{et al} assessed levels of methylation in 30 CpG islands from 50 CRCs.\textsuperscript{30} They found two patterns they designated type A (age-related), low level methylation that
increased incrementally with age, and type C (cancer-related) with high-level methylation of a distinct subset of CpG islands sufficient to result in gene silencing. Frequent type C methylation occurred in 27% of the CRCs and these were termed CpG island methylator phenotype (CIMP) tumours. Critically, the CIMP group encompassed virtually all cases of sporadic MSI-H cancers. This was accurately attributed to methylation induced silencing of MLH1, providing an explanation for MSI carcinomas occurring outside of Lynch syndrome.

Two major ‘CIMP panels’ are used for classifying CRCs and many groups use their own panels. This makes comparison of CIMP between studies difficult and is partly responsible for the reported variability in tumour numbers arising via the serrated neoplasia pathway. High rates of synchronous or metachronous MSI cancers in patients with serrated polyps and SPS, and CRCs arising in serrated-type polyps that exhibited CIMP, MLH1 silencing and MSI supported the existence of a serrated neoplasia pathway but one crucial molecular mechanism remained to be discovered.

MAPK PATHWAY ACTIVATION

The mitogen activated protein kinase (MAPK) pathway is a critical mechanism for cell signal conduction; mediating responses to extracellular signals relating to cell growth, differentiation and apoptosis. Mutations of components of this pathway have been identified in many types of cancer. Activation of BRAF or KRAS, components of the MAPK signaling cascade, in response to upstream signaling results in increased cell division and reduced apoptosis. Activating mutations of either BRAF or KRAS lead to constitutive activation of this pathway.

KRAS mutations, common in the chromosomal instability pathway, have also been identified in a subset of cancers arising via the serrated neoplasia pathway. Rajagopalan et al first described BRAF mutations in CRC in 2002. Importantly, they also found that BRAF and KRAS mutations are mutually exclusive and that BRAF mutations strongly correlate with both MSI and CIMP.

DEFINING THE SERRATED NEOLASIA PATHWAY
Drawing on the above information, the defining molecular features of the serrated neoplasia pathway are: 1) MAPK pathway activation, and 2) CIMP. MAPK pathway activation occurs primarily by either \( \text{BRAF} \) or \( \text{KRAS} \) mutation and CIMP can be either low level (CIMP-L) or high level (CIMP-H). Although important, MSI is not a requirement of the serrated neoplasia pathway.

The current model of serrated pathway carcinomas

As can be inferred from the above, carcinomas arising via the serrated pathway are a heterogenous group. In 2007 Jass proposed three broad molecular profiles for serrated pathway carcinomas, which have been modified slightly as follows:\(^1\)

1. CIMP-H, \( \text{BRAF} \) mutant
   a) MSI-H
   b) MSS

2. CIMP-L, MSS, \( \text{KRAS} \) mutant

Group one is the most strongly linked with the serrated neoplasia pathway. These cancers most likely arise in sessile serrated adenoma/polyps (SSAs) and are typically CIMP-H regardless of the panel used to define CIMP. Group two cancers are less strongly associated with the serrated neoplasia pathway, primarily because of a lack of consensus regarding what defines CIMP. They are postulated to arise in traditional serrated adenomas (TSAs).

Precursor lesions

Currently, three major categories of serrated polyp are recognised in the WHO classification; namely the HP, the SSA and the TSA.\(^37\) This classification is based predominately on the work of Torlakovic and Snover.\(^38\) However, there is continued debate regarding the diagnostic criteria and degree of malignant potential of these polyps.

HYPERPLASTIC POLYPS

HPs are common, accounting for 25-30% of resected large intestinal polyps.\(^39-43\) They have an estimated prevalence of 10-20% in Western adult populations.\(^44\) \( \text{BRAF} \) or \( \text{KRAS} \) are frequently mutated and are likely initiating events in the majority of HPs.\(^40\) Three HP subtypes are recognised, namely the microvesicular hyperplastic polyp (MVHP), the goblet
cell hyperplastic polyp (GCHP) and the mucin-poor hyperplastic polyp (MPHP). MVHPs and GCHPs are the most common while MPHPs are rare. The malignant potential of HPs is likely to be insignificant and under current guidelines no additional surveillance is required. 

**Microvesicular Hyperplastic Polyp**

Most MVHPs occur in the distal colon or rectum (74%), are asymptomatic and as such typically represent incidental findings during colonoscopy.

**Pathological Features**

At endoscopy most MVHPs are less than 5mm in diameter, light tan, small and flat (Figure 1). Magnification chromoendoscopy identifies stellate crypt openings (common to MVHPs and SSAs). Although large and/or proximal MVHPs may occur, the pathologist should look carefully in such lesions for features of SSA.

MVHPs are characterised by a serrated gland profile and an ordered ‘test-tube’ arrangement of the crypts that taper from the luminal to the basal aspect (Figure 2A). Serration is predominantly superficial and most cells have microvesicular mucin droplets that impart a hazy, basophilic quality to the cytoplasm (Figure 2B). The superficial subepithelial basement membrane and muscularis mucosae are thicker than in the adjacent normal mucosa and vertically oriented strips of smooth muscle are seen in-between crypts in some cases.

The proliferative zone of the crypts are symmetrically expanded compared with adjacent non-lesional mucosa and may show minor nuclear atypia, stratification and mitoses.

Immunohistochemistry shows regularly expanded proliferative and luminal compartments with Ki67 and CK20 respectively. p16 staining is frequently observed in the crypt bases and MVHPs are usually positive for the mucin core proteins MUC2 and MUC5AC and variably for MUC6.

**Molecular Features**
The fundamental molecular alteration in most MVHPs is the V600E \textit{BRAF} mutation\cite{40, 54} which induces constitutive activation of the MAPK pathway resulting in a burst of proliferative activity and inhibition of apoptosis. The process of activation-induced senescence is then postulated to curtail proliferation.\cite{55-58} Failure of apoptosis as the colonocytes reach the epithelial surface, with resultant epithelial crowding is thought to be responsible for the serrated morphology.\cite{59}

**Goblet Cell Hyperplastic Polyp**

GCHPs are typically diminutive lesions (92\% less than 5mm) and occur predominantly in the distal colon and rectum (68\%).\cite{41} Around half harbour a \textit{KRAS} mutation.\cite{41} Whether GCHPs have the ability to progress to carcinoma is not clear. By endoscopy GCHPs tend to be pale and sessile and are easily overlooked. The pathological features are similarly innocuous and the first impression is often of normal mucosa. Closer inspection reveals a thickened mucosa with crowded crypts containing a disproportionately high number of mature goblet cells (Figures 2C and D).\cite{38} Serration is often minimal or limited to the upper third of the crypt, but tufting of the epithelial surface is frequent (Figure 2D). Thickening of the basement membrane and muscularis mucosae is usually prominent.\cite{38}

**Mucin-Poor Hyperplastic Polyp**

These rare polyps probably are not a distinct entity, most likely representing a damaged MVHP.\cite{2} They share many histological features with MVHPs but show a relative lack of goblet cells and microvesicular mucin (Figures 2E and F).\cite{38} The superficial cells are low columnar or cuboidal, the nuclei are frequently hyperchromatic, and mild regenerative atypia may be present (Figure 2F).

**SESSILE SERRATED ADENOMA/POLYP**

Torlakovic and Snover coined the term SSA in 1996 to describe atypical HPs in patients with SPS.\cite{10} Their rates in colonoscopic series are highly variable (range 1.7\% to 9\% of all polyps).\cite{41, 60} The figure of 9\% was attained with colonoscopies performed by a single operator with meticulous technique and histology reviewed by a single expert pathologist and is likely to represent a close approximation to actual polyp prevalence in Western
centres. The SSAs mostly occurred proximally (75%) and showed considerable variation in size (36% ≤5mm, 47% 6-10mm and 17% ≥11mm).

The origin of SSAs is debated. We see SSAs with as few as four crypts, suggesting that at least some arise de novo. Supporting an origin from MVHPs are the histological similarities and common association with BRAF mutation; against are the markedly different distributions and the minimal malignant risk associated with HPs in population-based studies. There does appear to be a histological continuum from MVHP to SSA to SSA with dysplasia (SSAD) and finally to invasive carcinoma suggesting that these lesions represent a biological spectrum. A provisional category of borderline SSA has been proposed by some authors to describe a lesion intermediate between MVHP and SSA. The pathological features of the SSA, borderline SSA and SSAD are discussed separately below, followed by a unified discussion of their molecular features.

Pathological Features

By endoscopy SSAs are sessile and often yellow with a mucus cap that must be removed to allow adequate visualization; they also frequently show rims of bubbles and debris, alteration of fold contour and interruption of the underlying mucosal vascular pattern. Magnifying chromoendoscopy shows stellate crypt openings, and more recently, wider more rounded stellate pits have been described. Macroscopically most SSAs are subtle and easily missed on casual inspection, appearing as pale, ill-defined lesions that frequently extend over multiple mucosal folds (Figure 3A).

The most obvious features of SSAs are architectural. These include dilated crypts as well as crypts with horizontal growth of the bases in L or inverted T shapes along the muscularis mucosae (Figures 4A-C). Crypt spacing is typically irregular and crypt branching is frequent. Luminal serration usually extends into the basal third of the crypts (Figure 4C). Increased intraluminal and intracellular mucin is common (Figure 4B) and displaced crypts can be seen herniating into the submucosa (Figure 4D). At higher power, disturbance to proliferation and maturation is evident, with asymmetric proliferative zones and maturation towards both the luminal and basal aspects of the crypts. That is mature goblet and foveolar cells extending both towards the luminal and basal aspects of the crypt (Figure 4C). Dystrophic goblet cells are frequent. The cytology is typically
quite bland but a minor degree of nuclear atypia is allowable, particularly in the crypt bases.38

By immunohistochemistry the proliferative and mature compartments of the crypt are disorderly and frequently overlap.48 Similar to MVHPs, SSAs usually express MUC2, MUC5AC and basal MUC6.51-53 This degree of overlap makes mucin immunohistochemistry impractical for discriminatory purposes.52 Table 1 compares the histological and immunohistochemical features of MVHPs and SSAs.

The natural history of SSAs without dysplasia is not well defined.67 It has been estimated that 1 in 17 SSAs progress to malignancy, a malignant potential at least equivalent to a conventional adenoma.68 In a retrospective follow-up study of patients with SSAs, of those having another colonoscopy, all but one had further polyps/tumours (including one CRC) supporting surveillance colonoscopies in these patients.69

**Borderline sessile serrated adenoma/polyp**

Most SSAs are easily diagnosed but a subset with minimal changes occurs and the borderline between SSA and MVHP becomes blurred (Figure 4E). Some authors have proposed an intermediate category for these cases and publications pertaining to this issue are outlined in table 2.51, 65, 70 We follow the WHO guidelines stating that at least three crypts or two adjacent crypts showing SSA features are sufficient for the diagnosis of SSA.37 This seems a reasonable compromise but molecular and follow-up data supporting this and other positions is lacking.

**SESSILE SERRATED ADENOMA/POLYP WITH DYSPLASIA**

The SSAD is a critical lesion in the serrated neoplasia pathway, heralding an advanced phase in polyp progression. These lesions show an abrupt transition from otherwise typical SSA to cytologically dysplastic glandular epithelium (Figures 5A-C).62, 67 The natural history is not well defined but they are postulated to progress rapidly to malignancy2 based on their relative rarity compared to BRAF mutant, CIMP-H carcinomas and the relatively frequent finding of early invasive carcinomas in small polyps (Figures 3B and 5A).67 In a large population based study, 13.2% of SSAs showed dysplasia, accounting for 0.17% of all colorectal polyps.60 In a recent prospective audit of polyps at
our practice, SSADs represented a similar proportion of all colorectal polyps (unpublished data). They are more common in women (59%) and occur at a median age of 67.60

**Pathological Features**

The endoscopic appearances of SSADs have not been described, but given the small size of the dysplastic focus in the vast majority; they may indistinguishable from ordinary SSAs. In three small series of SSAs with dysplasia or early carcinoma the mean size of polyps was 8.5mm, 8.9mm and 11.3mm respectively, comparable to SSAs without dysplasia.62, 67, 71 Importantly, three of the polyps were 5mm or less in size, including one with invasive carcinoma.67

Two types of dysplasia are described.62, 71 So-called ‘conventional adenomatous dysplasia’ is similar to that seen in conventional adenomatous polyps (Figures 5A and F). It is characterised by increased nuclear pleomorphism, stratification and loss of polarity, atypical mitoses and basophilic cytoplasm with changes extending to the polyp surface; however in our experience some evidence of serration usually remains. Caution is warranted when assessing poorly oriented sections to prevent over-interpretation of the proliferative compartment as dysplastic. The second pattern is serrated dysplasia (Figures 5B-D, G and H).62 In this pattern the glands retain a serrated architecture, the cells have ample eosinophilic cytoplasm and the nuclei are typically vesicular and basally located. Not infrequently SSAs show focal ‘eosinophilic change’ resembling the eosinophilic cells in TSAs. This finding is often superficial and shows a gradual transition from the surrounding SSA. We do not consider this appearance sufficient to justify a diagnosis of SSAD.

Grading of dysplasia in serrated lesions is not advocated in the WHO classification, the rationale being that once dysplasia of any grade has developed, the polyp has declared itself as biologically aggressive.37 In many cases the SSA transitions abruptly to high-grade dysplasia (Figure 5A) but in other cases, clear demarcations between SSA, low-grade dysplasia and then high-grade dysplasia can be identified (Figures 5B-D).

**Molecular Features**

Similar to MVHPs, the initiating event in SSAs is thought to be a **BRAF** mutation, present in 70-81% of SSAs.41, 72 Methylation induced silencing of **p16** was proposed to allow progression from MVHP to SSA via escape from oncogene-induced senescence,73 but
more recently it has been shown that loss of p16 staining coincides with development of high-grade dysplasia or invasive carcinoma, late in polyp progression.\textsuperscript{49, 50} \textit{MLH1} silencing, one of the best-characterised features of SSAs, is restricted to SSADs\textsuperscript{29, 67} and loss of staining for the MLH1 protein is usually limited to areas of high-grade dysplasia (Figures 5D and E).\textsuperscript{62, 67}

Activation of the Wnt signaling pathway, typically associated with the chromosomal instability pathway, has also been implicated in the progression of SSAs.\textsuperscript{74} A proportion of SSADs show aberrant nuclear staining for $\beta$-catenin; however in nearly all instances the \textit{CTNNB1} gene (encoding $\beta$-catenin) is wild type, implicating upstream mechanisms for pathway activation.\textsuperscript{75} In particular, methylation of the Wnt pathway antagonists \textit{SFRP 1, 2 and 5} has been demonstrated in some SSAs.\textsuperscript{74} Methylation of the \textit{MGMT} gene, which codes for a DNA repair protein, has also been identified in a subset of SSAs and may be of particular relevance in \textit{BRAF} mutant MSS CRC.

Of note, nearly all the molecular changes described occur in advanced polyps. Currently only \textit{BRAF} mutations have been consistently identified in early SSAs, thus the molecular distinction between MVHP and early SSA remains unclear. We favour the hypothesis that MVHPs are unlikely to progress to SSAs unless critical molecular changes are acquired, such as methylation of key tumour suppressor genes.\textsuperscript{76} This is more likely to occur in the proximal bowel where CIMP is most common.

**TRADITIONAL SERRATED ADENOMA**

In 1990 Longacre and Fenoglio-Preiser described the ‘serrated adenoma’, a polyp characterised by serrated architecture and uniform, characteristic cytological atypia.\textsuperscript{77} Subsequent confusion with MVHPs, SSAs, SSADs and tubulovillous adenomas with serrated features (sTVAs) occurred and only after renaming as TSA by Torlakovic \textit{et al} in 2003, did it re-emerge as a distinct entity.\textsuperscript{38} TSAs are the least frequent serrated polyp accounting for around 1\% of colorectal polyps (range 0.6-1.9\%).\textsuperscript{41, 43, 77} They can occur throughout the large bowel but have a predilection for the distal colon and rectum.

\textit{Pathological features}
At endoscopy TSAs have a pinecone-like or raised two-tier appearance while magnifying chromoendoscopy shows a stellar or fern-like pit pattern.\textsuperscript{78} Macroscopically they may be either sessile (33\%), particularly proximally, or polypoid in appearance (67\%) (Figures 6A and B).\textsuperscript{79} Most exceed 5mm.

The histopathological features of TSAs are quite characteristic resulting in high diagnostic reproducibility.\textsuperscript{48} They typically display tubulovillous architecture, eosinophilic tall columnar epithelium with prominent serration (Figures 7A and B) and ectopic crypt foci (ECF) (Figure 7C).\textsuperscript{48} Serration in TSAs differs from that in MVHPs and SSAs, taking two forms: deep narrow ‘slit-like’ indentations from the luminal surface similar to those seen in normal small intestinal villi, more prominent in right sided \textit{BRAF} mutated lesions with SSA components (Figure 7A), and surface indentations associated with ECF (Figure 7C). ECF maintain their orientation towards the bowel lumen but lose their connection to the underlying muscularis mucosae, speculated to allow protuberant growth.\textsuperscript{48, 80} The villous tips in TSAs are frequently bulbous (tennis racquet-like) and sometimes linked by mucosal bridges.\textsuperscript{48} The characteristic epithelial cells have abundant pink cytoplasm and centrally located palisaded, pencillate nuclei with dispersed chromatin (Figure 7C);\textsuperscript{48, 77} frequently referred to as dysplastic, they may represent senescent cells.\textsuperscript{2} As similar cells can occur focally in MVHPs, SSAs, TVAs and regenerative epithelium,\textsuperscript{2} ECF are suggested as a more reproducible diagnostic feature.\textsuperscript{48} Mitoses are typically rare. Areas of goblet cell differentiation are fairly common in TSAs (Figure 7D) and in occasional cases goblet cells are the predominant cell type. Progression to high-grade dysplasia and carcinoma, usually with serrated morphology, is also not unusual (Figure 7E).

By immunohistochemistry the Ki-67 proliferative index is typically very low in eosinophilic cells but elevated in ECF; conversely CK20 staining is positive in the surface cells but not ECF.\textsuperscript{48}

\textit{Molecular Features}

Due to their rarity and confusion with other polyps, the molecular genetics of TSAs are poorly defined and studies are conflicting. Contrary to the current WHO classification,\textsuperscript{37} we and others see TSAs (particularly right-sided) with components of MVHP or SSA (Figure 7A),\textsuperscript{81} high rates of MLH1 methylation are present in these lesions, particularly when foci of high-grade dysplasia or malignancy are present. In the same series, \textit{KRAS}
and BRAF mutations were frequent (29% and 55% respectively), KRAS mutation and MGMT silencing (63%) were particularly common in advanced lesions, and high-grade serrated and conventional adenomatous dysplasia and invasive malignancy (26.7%, 18.8% and 8% respectively) were frequent; all carcinomas showed serrated morphology. CIMP-H has been reported in as many as 79% of TSAs by some but is much lower in other series, the variance likely reflecting discrepancies in histological diagnosis and the panels of methylation markers used to define CIMP.82, 83

Filiform Serrated Adenoma

Filiform serrated adenoma83, 84 accounts for 4% of TSAs, occurs distally and is characterised by very long (filiform) villi (Figure 8A) with marked lamina propria oedema (Figures 8A and B) and frequent epithelial erosions. The epithelium is typically an admixture of eosinophilic cells and goblet cells as seen in TSAs. Twenty-two percent show high-grade dysplasia and 6% contain a focus of adenocarcinoma.

The immunohistochemical and molecular features appear to be similar to TSAs. In one series, BRAF or KRAS mutations occurred in 71%, MSS in 58% and MSI-L in 42%; MSI-H and loss of MLH1 was not seen.84 In another, all lesions were MSS, CIMP-H occurred in 38% and most of the remainder were CIMP-L.83

Although filiform serrated adenomas appear pathologically and molecularly similar to TSAs, we have also seen filiform change in TVAs. We believe this change is the result of polyp trauma and prolapse.

Tubulovillous adenoma with serration

In our experience, serrated TVAs (sTVAs) are common and their histological appearances overlap with those of TSA. They may also have molecular features such as a high frequency of KRAS mutation, more in common with TSA than conventional adenoma.85 As we not infrequently see serrated-morphology CRC arising in sTVAs, we propose that sTVA, along with TSA, may be the precursors of this group of CRC. Detailed histological, immunohistochemical and molecular evaluation of this potential alternate precursor to the serrated pathway is required.
FIBROBLASTIC POLYP

The fibroblastic polyp is intimately associated with MVHPs and SSAs. First described in 2004, they are typically small and distal. Although subsequently demonstrated to be mucosal perineuriomas, the term fibroblastic polyp remains entrenched in the literature. Fibroblastic polyps are benign and do not require any specific follow-up.

Histologically they show expansion of the lamina propria by a bland spindle cell proliferation with a pushing margin (Figures 9A and B). Superficially the uniform spindle cells with oval nuclei lie parallel to the luminal surface. Elsewhere they are less orderly but frequently whorl around vascular and epithelial structures. A component of fibroblastic polyp is reported in 6.5% of SSAs; this frequent occurrence is in line with our experience. The spindle cells stain positively for vimentin, EMA (often weak), Glut-1, collagen IV and Claudin-1, which is typical of perineurial differentiation. BRAF mutations occur in the associated serrated epithelial component, demonstrating that the epithelium is neoplastic rather than reactive in nature. It is suggested the cells are fibroblasts that have undergone perineurial differentiation, possibly induced by the associated serrated polyp epithelium.

MIXED POLYPS

Mixed polyp has been used to describe any number of polyp combinations including, until recently, SSADs. As such the term has been a source of confusion and has been removed from the current WHO classification. Despite this, ‘mixed’ polyps’ not otherwise accounted for in the classification do occur (Figures 10A and B). In these instances we name the component parts rather than use the unqualified term ‘mixed polyp’.

Serrated neoplasia pathway carcinomas

Serrated pathway carcinomas with the three broad molecular profiles of Jass are now discussed.

1. a) BRAF MUTANT / CIMP-H / MSI-H
Accounting for 9-12% of CRC and constituting the ‘classic’ tumours derived from the serrated neoplasia pathway,¹ this group tends to occur in elderly women and has a marked predilection for the right colon. Frequently they present with high tumour stage but without nodal or distant metastases. Residual SSA may rarely be identified adjacent to the cancer. The histology is well characterised and includes pushing margins, poor or mucinous differentiation, peri-tumoural Crohn’s-like inflammatory infiltrate, tumour infiltrating lymphocytes and a clonal growth pattern.⁹¹ Many of these features seem to be related to MSI and as such are shared with Lynch syndrome tumours.⁹², ⁹³ The precursor SSA undergoes progressive methylation of key promoter regions, in particular MLH1, the silencing of which typically coincides with development of high-grade cytological dysplasia and MSI.⁹⁴ The acquisition of these genetic changes may herald a rapid progression to malignancy and subsequently account for a disproportionate number of interval carcinomas.², ⁹⁵ The tumours have a favourable prognosis compared to stage matched controls but are resistant to most non-surgical treatments, including 5-fluorouracil (5-FU) and the monoclonal epidermal growth factor receptor (EGFR) inhibitors, cetuximab and panitumumab.⁹⁶, ⁹⁷ Identification of mutant BRAF in these cancers excludes Lynch syndrome.

1. b) BRAF MUTANT / CIMP-H / MSS

Sessile serrated adenomas with dysplasia but without loss of MLH1 expression are the proposed precursors of this subgroup and account for 6-8% of CRCs. They are also more frequent in the right colon.¹ The carcinomas are often poorly differentiated and mucinous.⁹⁸ Signet ring cell morphology and the rare cribriform-comedo subtype have been associated with this molecular category.⁹⁹ These tumours have higher rates of tumour budding, lymphatic, vascular and perineural invasion and lymph node metastases than other CRCs.⁹⁸ The accumulation of genetic abnormalities in these cancers also occurs via CIMP with methylation of different gene promoters than in MSI-H cancers; silencing of p16 and Wnt pathway genes has been proposed.¹ Mutation of p53 is more common compared with BRAF mutant MSI-H carcinomas.¹⁰⁰ In most series they have a poor prognosis.⁹⁸, ¹⁰¹, ¹⁰²

2. CIMP-L / MSS / KRAS MUTANT
This is potentially the largest and certainly the most controversial group and may account for 15-20% of all CRCs. They are generally thought to arise in TSAs, but as these adenomas are relatively rare, there must be other precursors that we suggest are sTVAs progressing via a serrated-ike pathway. Although there is currently no marker panel available to specifically detect CIMP-L, the genetic changes of \textit{KRAS} mutation and \textit{MGMT} methylation frequently segregate with CIMP-L cancers, suggesting they form an important molecular subgroup. Because these tumours have not been clearly defined, there is limited clinicopathological data pertaining to them.

Comparison of the serrated neoplasia molecular pathways with other putative molecular pathways to CRC is shown in Figure 11.

**Serrated morphology carcinomas**

The term ‘serrated carcinoma’ has been used to refer to all tumours arising via the serrated neoplasia pathway as well as to describe a subset of cancers with a distinctive serrated morphology. In this review we use the term ‘serrated pathway carcinoma’ for all serrated pathway cancers (regardless of morphology) and ‘serrated morphology carcinoma’ for the histologically distinct serrated subset. The WHO classification now recognises serrated morphology carcinomas as a CRC subtype. These carcinomas were first described by Jass and the histological criteria refined by Makinen and Tuppurainen et al. Seven to twelve percent of CRCs have serrated morphology. Given that 30% of CRC is attributed to the serrated neoplasia pathway, then about one third of these cancers display serrated morphology.

Serrated morphology carcinomas are more frequent in women with over half arising in the caecum or ascending colon and about one third in the rectum. No specific macroscopic features have been described. Three major histological patterns are recognised. Well to moderately differentiated adenocarcinoma with prominent serrations comprising epithelium only or epithelium with basement membrane is most common (Figures 12A and B). The epithelial cell cytoplasm is typically intensely eosinophilic and abundant and the nuclei vesicular and basal (Figure 12C). Necrosis is usually absent and mucinous differentiation at the deep aspect of the tumour common. An unequivocal diagnosis of serrated carcinoma is said to require at least six of the first seven features listed in Table 3; cases with only five features or with 10-20% necrosis are considered equivocal.
The second pattern, accounting for 20% of cases is mucinous adenocarcinoma (Figure 12D). Extracellular mucin constitutes at least 50% of the cross-sectional area and by definition they are poorly differentiated. Serrations can usually be identified, at least focally, and typical serrated carcinoma cytology is retained. Cell balls and papillary rods floating in the mucin are common and characteristic of this pattern (Figure 12E).

The final pattern is trabecular, constituting around 7% of cases. These tumours are poorly differentiated with tumour cells growing in a trabecular fashion; epithelial serrations are typically lost (Figure 12F). Micropapillary structures are sometimes seen and lymphatic invasion is common. Serrated morphology carcinoma cytology remains apparent. This pattern is often a minor component of other subtypes, usually at the advancing edge of the tumour.

Serrated morphology carcinomas segregate as a distinct molecular subset, strengthening the assertion they represent a reproducible and distinctive subtype of CRC. KRAS and BRAF mutations have been identified in 45% and 33% of cases respectively; MSS in 50%, MSI-H in 16% and MSI-L in 30%. These figures suggest that serrated pathways 1b and 2, described above, are over-represented.

More recently serrated morphology carcinomas have been shown to have a poor prognosis compared to stage-matched conventional-type CRC. Tumour budding, infiltrative growth pattern and lymphatic invasion are all more frequently identified in these tumours and are likely to be contributive.

Serrated morphology carcinomas in our experience arise from TSAs and sTVAs. Refinement of diagnostic criteria for these carcinomas with a greater emphasis on histological and molecular features and less on origin from a serrated polyp may result in more robust clustering of these cancers with even greater prognostic relevance.

**Treatment issues relevant to serrated pathway carcinomas**

Although 5-FU with either oxaliplatin or irinotecan remains the mainstay of medical treatment in advanced stage CRCs, targeted treatment options are now in clinical practice or being evaluated in trials. Monoclonal EGFR inhibitors are currently widely
used. The EGFR signaling pathway, involved in cellular proliferation and apoptosis via either the MAPK or phosphatidylinositol 3-kinase (PI3K) pathways, is integral to serrated neoplasia. Blocking the EGFR receptor has proven effective\textsuperscript{114} but mutations in downstream effectors of the EGFR pathway block tumour response. The best characterised are activating \textit{KRAS} mutations, seen in 30-40\% of CRCs.\textsuperscript{115} As such \textit{KRAS} mutation testing is mandatory prior to treatment with these expensive and potentially toxic medications.

\textbf{BRAF} acts immediately after \textit{KRAS} in the MAPK signaling cascade and activating \textit{BRAF} mutations, seen in about 10\% of CRCs, could also nullify the benefits of anti-EGFR therapy; however unresponsiveness is less obvious than for \textit{KRAS} mutant tumours.\textsuperscript{97} In the alternate EGFR signaling pathway PI3K activation and PTEN inactivation also confer resistance to anti-EGFR therapy and may account for a proportion of non-responders with wild type \textit{KRAS} and \textit{BRAF}.\textsuperscript{97}

Following from its success in metastatic melanoma, the V600E mutant specific, BRAF inhibitor vemurafinib has been trialed in CRC.\textsuperscript{116} Unfortunately less than 5\% of \textit{BRAF} mutant CRCs demonstrate a response, likely secondary to rapid feedback activation of EGFR.\textsuperscript{116} Cell lines dual treated with vemurafinib and an EGFR inhibitor showed substantial tumour suppression\textsuperscript{116} and clinical trials using dual therapy are planned.

\textbf{Future directions}

There has been enormous progress in our understanding of the serrated neoplasia pathway but many fundamental issues remain unresolved. The histological distinction of MVHP from SSA with minimal features is subjective and lacks reproducibility. Also there are no histological or molecular markers to identify a SSA that is likely to progress from one which will remain indolent. Although now generally accepted that a SSAD is an aggressive lesion, the significance of subtle serrated dysplasia is unknown.

From a clinical perspective these issues are frustrating. MVHPs do not require additional surveillance, whereas patients with SSAs are typically surveilled at the same rate as conventional adenomas. SSADs are often managed aggressively, coming to colectomy if complete endoscopic resection cannot be achieved. Thus accurate and reproducible diagnosis of serrated polyps is imperative. Furthermore, long-term prospective studies
addressing outcomes after diagnosis of the various types of serrated polyp will be critical to developing reliable surveillance guidelines for these lesions.

There also remain gaps in our knowledge of the underlying molecular mechanisms of the serrated pathway. The primary cause of CIMP remains unclear. Although irrefutably correlated, no mechanistic relationship between CIMP-H and \textit{BRAF} mutation has been established. While CIMP-H CRC is an established entity, there is continued debate as to the significance of CIMP-L.

Although the classification of Jass\textsuperscript{1} has proved useful, serrated pathway carcinomas are proving more heterogeneous than expected. As the repertoire of targeted therapies expands, there is likely to be increasing emphasis on the underlying molecular alterations in CRCs. Serrated pathway cancers are particularly prone to mutations in pathways targeted by these agents.

Finally the ‘$1000$ genome’ is likely to become a reality in the next few years. Next generation sequencing offers the opportunity to completely profile the genome, methylome and transcriptome of a CRC. Mining this data will be an enormous bioinformatics challenge but will doubtless yield an array of potential biomarkers and targets for molecular-based therapy.

\textbf{Conclusions}

The serrated neoplasia pathway is a critical route to CRC. Furthermore it serves as a model of the importance of epigenetics in carcinogenesis. In the last few years most pathologists and clinicians have become comfortable with the concept of an alternative pathway to CRC but many diagnostic and prognostic issues persist. The imminent arrival of complete genetic profiling of tumours will create an enormous amount of data pertaining to all types of cancer. However, interpretation of this information can only be informative in combination with accurate clinical, pathological and follow-up information. The pathologist is suitably positioned to integrate pathological and molecular information into a single comprehensive report. Thus it will be incumbent on pathologists to keep themselves abreast of the important information these evolving molecular techniques provide.
References


### Table 1. Comparison of MVHP and SSA.

Note that architectural features are the most discriminatory.
<table>
<thead>
<tr>
<th>Study</th>
<th>Polyp category</th>
<th>Diagnostic criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chung et al</td>
<td>SSA</td>
<td>- Polyp &gt;10mm&lt;br&gt;- Polyp proximal to the hepatic flexure and&lt;br&gt;- At least four of exaggerated serration, crypt dilation, increased crypt branching/horizontal growth, cytological atypia, mitoses in upper half of the crypt, increased cytoplasmic mucin and epithelial:stromal ratio of &gt;50%</td>
</tr>
<tr>
<td>Intermediate between MVHP and SSA</td>
<td>- Polyp &lt;10mm&lt;br&gt;- Polyp anywhere in the large bowel&lt;br&gt;- At least four of the above criteria</td>
<td></td>
</tr>
<tr>
<td>MVHP</td>
<td>- Three or fewer of the above criteria</td>
<td></td>
</tr>
<tr>
<td>Mohammadi et al</td>
<td>SSA</td>
<td>- At least two of basal crypt dilation, basal crypt serration, crypt branching or horizontal crypt growth</td>
</tr>
<tr>
<td>Borderline SSA</td>
<td>- Only one of the above criteria, or&lt;br&gt;- Equivocal evidence of two of the above criteria</td>
<td></td>
</tr>
<tr>
<td>MVHP</td>
<td>- None of the above criteria, or&lt;br&gt;- One equivocal criterion</td>
<td></td>
</tr>
<tr>
<td>WHO 2010</td>
<td>SSA</td>
<td>- At least two adjacent crypts or three individual crypts with features of SSA</td>
</tr>
<tr>
<td>MVHP</td>
<td>- Not meeting above criteria</td>
<td></td>
</tr>
<tr>
<td>Aust et al</td>
<td>SSA</td>
<td>- Two of basal crypt serration, horizontal crypt growth, inverted crypts and basal crypt dilation&lt;br&gt;- Above features in at least two crypts</td>
</tr>
</tbody>
</table>
Table 2. Differing diagnostic criteria for SSA, borderline SSA and MVHP
1. Epithelial serrations  
2. Eosinophilic or clear cytoplasm  
3. Abundant cytoplasm  
4. Vesicular nuclei with peripheral chromatin condensation and a single prominent nucleolus  
5. Distinct nucleoli  
6. Absence of necrosis (or less than 10% necrosis)  
7. Intracellular and extracellular mucin  
8. Cell balls and papillary rods*  

Table 3. Histological criteria of serrated morphology carcinomas.  
*Typically only seen in mucinous carcinomas
Figure legends

Figure 1. Hyperplastic polyps. (bar = 20mm).

Figure 2. Hyperplastic polyps. A and B. MVHP. B. Microvesicular mucin droplets and superficial goblet cells. C and D. GCHP showing crowded crypts, dominance of goblet cells and minimal superficial serration (D). E and F. MPHP with cuboidal to low columnar epithelial cells, mucin depletion, lack of goblet cells and fine ‘saw-tooth’ serration.

Figure 3. A. Sessile serrated adenoma. Arrows mark edges of SSA. B. SSA with early carcinoma. The erythematous/ulcerated area represents the early carcinoma; the arrows indicate the edges of the residual SSA. (bars = 20mm).

Figure 4. A-D. Sessile serrated adenoma. A. Typical SSA showing dilated crypts with horizontal growth along the muscularis mucosa and deep serration. B. Alternate pattern with predominance of dilated crypts with abundant luminal mucin. C. Same polyp as A showing asymmetrical proliferative zone and maturation extending into the deep aspect of the crypt characterised by goblet cells in the crypt base. D. Inverted growth pattern. E. So-called borderline SSA with minimal basal crypt dilation and deep serration. The features are intermediate between a MVHP and a SSA. By default we revert to a diagnosis of HP in these cases.

Figure 5. A-H. Sessile serrated adenomas with dysplasia. A. Abrupt transition from SSA (*) to high-grade adenomatous dysplasia (***) and early carcinoma (**). Note the loss of the muscularis mucosa at the transition to early invasive carcinoma (arrow). B-D. A large SSA showing abrupt transition from SSA to low-grade serrated dysplasia (lower right arrow and image C) and then to high-grade dysplasia (upper left arrow and image D). The high-grade dysplasia in this case is not typical of either true adenomatous or serrated type dysplasia. E. Abrupt loss of MLH1 staining at the transition from low-grade serrated dysplasia to high-grade dysplasia (immunohistochemistry for MLH1). F-G. Cytological features of high-grade adenomatous dysplasia (F), low-grade serrated dysplasia (G) and high-grade serrated dysplasia (H).

Figure 6. Traditional serrated adenomas from the sigmoid colon. A. Sessile TSA. B. Polypoid TSA. (bars = 10mm).
**Figure 7.** A-E. Traditional serrated adenomas. A. TSA from transverse colon with sessile growth pattern and component of SSA, in keeping with proximal location and *BRAF* mutation. (A *BRAF* V600E mutation was confirmed in this polyp). B. Rectal TSA with polypoid growth pattern in keeping with distal location. C. Characteristic cytological features (abundant eosinophilic cytoplasm and centrally placed, pencillate nuclei) and ECF (examples indicated by arrows). D. Transition from typical to goblet cell predominant pattern of TSA (same polyp as 7B). E. High-grade dysplasia in an otherwise typical TSA. Note retention of ECF.

**Figure 8.** A and B. Filiform serrated adenoma. Note elongated ‘filiform’ processes (A) with oedematous tips (B).

**Figure 9.** A and B. Fibroblastic polyp arising in a SSA. Note bland spindle cells with eosinophilic cytoplasm encompassing crypts.

**Figure 10.** A and B. Mixed polyps. A. MVHP (right) and TSA (left). B. MVHP (left) and tubular adenoma with low-grade dysplasia (right)

**Figure 11.** Putative pathways to colorectal cancer.

**Figure 12.** A-F. Serrated morphology adenocarcinomas. A and B. Well-differentiated adenocarcinoma with prominent epithelial serrations. C. Typical cytology with abundant eosinophilic cytoplasm and vesicular, basal nuclei. D and E. Mucinous adenocarcinoma. This arises in the SSAD depicted in Figure 5B. E. Cell balls and papillary rods floating in mucin. F. Trabecular adenocarcinoma. Micropapillary clusters and signet ring cells are also evident.

**Acknowledgments:**
The Cancer Council Queensland for PhD scholarship funding to Mark Bettington
PUTATIVE MOLECULAR PATHWAYS TO COLORECTAL CARCINOMA

Serrated pathways

Normal mucosa

BRAF, CIMP-H

SSA

MLH1 loss

p16 loss

MGMT loss

SSAD

MSI (frameshift mutations e.g., TGFβ/III (GFIIR))

BRAF, CIMP-H, MSI CRC

Good prognosis

Resistant to SFU

Resistant to anti-EGFR therapy

Poor prognosis

Sensitive to SFU

Sensitive to anti-EGFR therapy

Conventional pathways

Normal mucosa

APC

TA

Hypomethylation

TA HGD

SMAD4, p53

CIMP-H, MSS CRC

Standard prognosis

Sensitive to SFU

Sensitive to anti-EGFR therapy

Familial pathways

Lynch (germline mutation of a MMR gene)

KRAS

APC

Loss of remaining MMR allele, p53

Hundreds of TAs

Hypomethylation

TA HGD

SMAD4, p53

CIMP-H, MSS CRC

Standard prognosis

Sensitive to SFU

Sensitive to anti-EGFR therapy

TVA HGD

KRAS, CIMP-L, MSS CRC

Standard prognosis

Sensitive to SFU

Resistant to anti-EGFR therapy
Conclusions
The serrated neoplasia pathway is relatively recently described and is the subject of ongoing intensive research. A remarkable amount has been achieved regarding the pathology and molecular biology of serrated polyps and carcinomas; however much remains to be resolved. It is clear that there are ongoing issues relating to the histopathological diagnosis of the sessile serrated adenoma and its troublesome distinction from microvesicular hyperplastic polyps. The biological progression of these polyps also requires clarification. It is increasingly apparent that traditional serrated adenomas can arise in sessile serrated adenomas, but the implications of this event are unclear. Furthermore, the frequency of \( \text{MLH1} \) methylation in sessile serrated adenomas with dysplasia is not clear and the implications for the resultant polyps and cancers are not completely understood.

The traditional serrated adenoma remains something of an enigma. Although thought to be quite simple to diagnose, the distinction from a subset of tubulovillous adenomas can be challenging. In particular the presence of ectopic crypt formations may not be as definitive in the diagnosis of traditional serrated adenomas as was previously believed. A lack of uniform diagnosis may underscore the variability in molecular biological features described for the traditional serrated adenoma. In particular the \( \text{BRAF} \) and \( \text{KRAS} \) mutation status and the CpG island methylator phenotype status vary widely in the literature. In addition detailed study of traditional serrated adenomas developing discrete areas of overt cytological dysplasia are limited and typically include few cases. Thus the molecular biology involved in the progression of traditional serrated adenomas is not clear and the nature of the cancers they give rise to has been inadequately addressed.
Hypotheses:

With the above framework in mind, we plan to clarify and build upon the current model of serrated colorectal polyps. My hypotheses are as follows.

1. Serrated polyps, in particular sessile serrated adenomas, are more common than is currently reported in the literature
2. A single unequivocal sessile serrated adenoma-type crypt is sufficient for diagnosis
3. There are histological, immunohistochemical and molecular features that can predict aggressive biology in traditional serrated adenomas and sessile serrated adenomas
4. A subset of tubulovillous adenomas with serrated histology can progress to malignancy via the serrated pathway
5. Serrated polyps represent distinct precursors of the molecular subtypes of CRC
Aims:

1. To develop a large cohort of consecutive polyps to provide information regarding the prevalence of serrated polyps in our community
2. To attempt to determine an appropriate diagnostic threshold for the sessile serrated adenoma based on a detailed clinicopathological assessment
3. To determine the *BRAF*, *KRAS*, CIMP and immunohistochemical status of histologically well-categorised cohorts of serrated polyps
4. To provide a detailed clinicopathological and molecular analysis of large cohort of sessile serrated adenomas and traditional serrated adenomas
5. To demonstrate molecular similarities between a subset of TVAs and polyps of the serrated neoplasia pathway (in particular the TSA)
6. To determine the polyp precursors of the different molecular subtypes of serrated pathway colorectal carcinomas
Chapter 2: Critical appraisal of the diagnosis of the sessile serrated adenoma

As published in American Journal of Surgical Pathology as:


Relevance to the aims of the thesis:

This chapter addresses thesis aims 1 and 2. The frequency of sessile serrated adenoma diagnoses in the pathology literature is quite conflicted with rates ranging from 1.1-10.1%.¹⁻⁵ This broad variance likely reflects a range of factors, including the patient population, skill and experience of the colonoscopists and the diagnostic criteria of the reporting anatomical pathologist.⁶ In our experience, using current diagnostic criteria, we felt that sessile serrated adenomas were more common than was being reported in the literature. If true, this has significant clinical implications. A low rate of sessile serrated adenoma diagnoses may reflect either failure to remove the lesions at colonoscopy, or under-reporting as microvesicular hyperplastic polyps by the pathologist. Both of these scenarios carry a risk of interval colorectal carcinoma, defined as the development of carcinoma within the colonoscopic surveillance interval. Interval colorectal cancers represent approximately 5% of all colon cancer diagnoses and serrated pathway cancers are over-represented in this group.⁷, ⁸ Thus high quality colonoscopy and accurate pathological diagnosis of sessile serrated adenomas should help reduce interval colorectal carcinoma.

4. Carr NJ, Mahajan H, Tan KL, Hawkins NJ, Ward RL. Serrated and non-serrated polyps of the colorectum: their prevalence in an unselected case series and correlation of


Critical appraisal of the diagnosis of the sessile serrated adenoma

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Running Title: Diagnosis of the Sessile Serrated Adenoma

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Conflicts of Interest: None to disclose
ABSTRACT
The sessile serrated adenoma (SSA) is a relatively recently described polyp that can present diagnostic difficulties for the practicing pathologist. The frequency of SSA diagnoses varies dramatically in the reported literature. In addition the histological interface between the microvesicular hyperplastic polyp (MVHP) and the SSA continues to be a diagnostic problem. The trend in recent years has been to a lower threshold for SSA diagnosis. Herein we have performed a cross-sectional study of 6340 colorectal polyps received at a high volume community-based pathology practice over a three-month period. After central review, with strict application of the diagnostic criteria outlined in the 2010 edition of the WHO Classification of Tumours of the Digestive Tract, we found that SSAs represented 12.1% of all polyps. In addition we developed novel diagnostic subcategories in an attempt to determine the most appropriate cut-off for the interface between the MVHP and the SSA. We found that serrated polyps (MVHPs or SSAs) with any SSA-like crypts had clinical features more in common with the SSA than the MVHP and that this diagnostic cut-off showed good reproducibility between pathologists. This supports the position of a recent consensus publication proposing that polyps with as few as one SSA type crypt should be diagnosed as a SSA. Applying these criteria to our cohort yield an overall SSA rate of 14.7%. In summary, we believe that SSAs continue to be under-diagnosed in pathological practice and that this may result in inadequate surveillance and thus contribute to interval colorectal carcinomas.

Key Words: Colorectal neoplasms; Colorectal polyps; Sessile serrated adenoma; Microvesicular hyperplastic polyp; Diagnosis
INTRODUCTION

Colorectal carcinoma (CRC) is the second leading cause of cancer death in the developed world. Approximately 20-30% of CRC occurs via the serrated neoplasia pathway\(^1\). Most of these cancers are thought to have their origins in a relatively recently described polyp, the sessile serrated adenoma/polyp\(^2\)-\(^5\). For simplicity we will use the term sessile serrated adenoma (SSA) throughout this paper.

Despite its well-documented importance as a precursor lesion of CRC, the proportion of colorectal polyps that are SSAs is currently unclear. A review of the literature identified eleven papers that addressed this issue, in which SSAs ranged almost ten-fold from 1.1\%-10.1\%\(^6\)-\(^16\).

The reported variability may result because serrated polyps occur on a diagnostic spectrum with plain microvesicular hyperplastic polyp (MVHP) at one end and frank SSA at the other. A diagnostic ‘grey-zone’ exists in the middle. In the 4th edition (2010) of the WHO Classification of Tumours of the Digestive System, guidelines were given for the diagnosis of the SSA, stating that if a serrated polyp shows two or three contiguous SSA-type crypts then a diagnosis of SSA can be made\(^17\). More recently an expert panel including gastroenterologists, scientists and pathologists published a consensus document pertaining to serrated polyps\(^18\). In particular they made the recommendation that serrated polyps with as few as one SSA-type crypt should be diagnosed as a SSA. Inevitably this has resulted in some confusion amongst pathologists as to the most appropriate cut-off for the diagnosis of a SSA. Some pathologists may have more readily adopted the recommendations of the consensus document, whereas other pathologists may not have been aware of the consensus document or may be reluctant to change their diagnostic criteria without supporting evidence.

Thus the primary aim of this study was to provide comprehensive colorectal polyp rates in a community-based, Australian gastrointestinal pathology practice with a particular emphasis on the contribution of the SSA. We secondarily sought to evaluate the guidelines for SSA diagnosis as provided in the WHO Classification and the recent consensus paper, by dividing all MVHPs and SSAs into subgroups based on the extent of colonic crypt abnormalities. Finally, we sought to validate the newly determined cut-off for the diagnosis of a SSA by conducting a reproducibility study using our revised diagnostic criteria.
We found that SSAs, as currently defined by the WHO, represented 12.1% of all colorectal polyps in this large, prospective series. Furthermore, we demonstrated that serrated polyps with only one abnormal SSA-type crypt had clinical features more closely related to typical SSAs than the plain MVHP, thus supporting a further relaxation of the current diagnostic criteria of the SSA as proposed in the consensus document. This new cut-off for SSA diagnosis showed good interobserver agreement amongst the study pathologists.

This trend to increasing SSA diagnoses is of great importance to patients, clinicians, researchers and health economists. In contrast to MVHPs, SSAs are recognised premalignant lesions\textsuperscript{2,4,5}. Over-diagnosis of SSAs results in unnecessary, invasive and costly endoscopic surveillance, whereas under-diagnosis may contribute to interval colorectal carcinoma.

**MATERIALS AND METHODS**

**Case collection and pathological review**
Colonic polyp tissue specimens were collected in a prospective fashion at a single institution (Envoi Specialist Pathologists) in Brisbane, Australia over a three-month period from the 30\textsuperscript{th} of January to the 29\textsuperscript{th} of April 2012. Envoi Specialist Pathologists is a community-based, gastrointestinal histopathology practice that receives specimens from over 60 gastroenterologists and surgeons. Only polyps identified endoscopically were included in the study. This study was approved by the institutional review board of The Queensland Institute of Medical Research.

Demographic data were collected from the information provided on the pathology request form. Polyp location was determined from the colonoscopy report when available or from the request form and specimen container label. Location was divided into proximal, distal and rectal. Proximal location was defined as cecum, ascending colon and transverse colon; distal location as the splenic flexure, descending colon and sigmoid colon. In statistical analyses rectal location was included in the distal category.

Where possible, the polyp size and number was extracted from the colonoscopy report. When endoscopic correlation was not possible, separate fragments were reported as a single polyp if all pieces were histologically similar and as separate polyps if different polyp
subtypes were evident within the separate tissue fragments. Because of the unreliable nature of estimating polyp size from the fragmented tissue received in the laboratory, size measurements were based only on endoscopic reports.

All polyps were initially reviewed by a single pathologist (MB). To simulate normal reporting conditions, the review diagnoses were based only on the material available to the original reporting pathologist, that is, three haematoxylin and eosin stained sections for each polyp along with any additional levels or stains performed during the reporting process. No additional levels or stains were performed for the purposes of review.

When applicable, the polyp diagnoses were based on the current WHO criteria\textsuperscript{17}. Diagnostic categories included conventional adenomas (including tubular adenoma, tubulovillous adenoma and villous adenoma), serrated polyps (including microvesicular, mucin-poor and goblet cell hyperplastic polyps, sessile serrated adenoma, sessile serrated adenoma with dysplasia, traditional serrated adenoma and serrated polyp unclassifiable) and others (including a variety of non-neoplastic and non-epithelial lesions). Polyps falling into the ‘other’ category were not included in the analysis.

Conventional adenomas were diagnosed on the combination of conventional adenomatous dysplasia and proportion of villous component. As per the WHO classification\textsuperscript{17}, cases with 0-25% villosity were diagnosed as TA, 25-75% villosity as tubulovillous adenomas (TVA) and >75% villosity as villous adenomas (VA). The diagnosis of a SSA was based on the criteria from the 4th edition of the WHO Classification of Tumours of the Digestive System\textsuperscript{17} which states that two or three contiguous SSA-type crypts is sufficient for the diagnosis of SSA. An SSA-type crypt is described as “dilated and assume abnormal shapes including L-shapes and inverted T-shapes. Serration may be very prominent and is often seen at the base of the crypts, rather than superficially as for HPs.”.

For the purposes of this study we sought to more precisely define crypt architectural changes that are diagnostic of SSA as; 1) any horizontal growth along the muscularis mucosae, 2) dilation of the crypt base (basal third of the crypt) such that it is wider than the luminal opening, 3) serration extending into the crypt base or 4) asymmetric proliferation (figure 1). Serrated polyps displaying any of these features in at least two contiguous or three non-contiguous crypts were classified as SSAs. Serrated polyps not meeting these
minimum criteria were classified as MVHPs. Because prolapse can produce similar crypt abnormalities, evidence of significant prolapse effect precluded the diagnosis of a SSA-type crypt. This was recognised by either smooth muscle proliferation in the lamina propria or by the presence of diamond-shaped crypts as recently described by Huang et al\textsuperscript{19}. Cytological features were not used to discriminate MVHP from SSA.

The diagnosis of sessile serrated adenoma with cytological dysplasia (SSAD) required an abrupt transition from typical SSA to overt cytological dysplasia within a single tissue fragment.

**Subdivision of MVHPs and SSAs**

For the second part of the study, all MVHPs and SSAs were further subdivided into a total of seven subcategories dependent on the number of SSA-type crypts (table 1 and figure 2). This subdivision was based only on the histology of the individual polyps and was blinded to all clinical and demographic data. When a diagnosis could not be achieved, typically because of poor section orientation that did not display the bases of the crypts, a designation of serrated polyp unclassified (SPUC) was rendered. The provisional SSA (pSSA) type 3 group represented polyps in which there were three or more crypts with equivocal SSA-type changes, that is crypts in which the base was dilated compared to the mid-crypt but of similar width to the luminal aspect or if there was undulation of the epithelium in the basal aspect of the crypt but without the tufted appearance seen in a typical SSA-type crypt (figure 2). Horizontal growth along the muscularis mucosa and asymmetric proliferation were not permitted in this group.

**Diagnostic Concordance Between Pathologists**

To assess diagnostic reproducibility, 30 consecutive MVHP, 30 consecutive pSSA types 1-3 and 30 consecutive SSA types 1-3 were selected from the series, giving a total of 90 polyps for review. A panel of four gastrointestinal pathologists (MB, NW, CR, IB) independently reviewed these polyps blinded to all clinical information and based their diagnoses only on the criteria outlined above and in table 1. These review diagnoses were required to be either MVHP or SSA (encompassing pSSA types 1-3 and SSA types 1-3).

**Statistical Analysis**

These groups were then subjected to statistical analyses to validate the new WHO diagnostic criteria. To remove the confounding effect of person, the presence or absence
of each polyp within each person was analysed. To test whether a particular type of polyp was more likely found proximal or distal/rectal, every person with at least one of the polyp type of interest was recorded as having their polyp(s) proximal, distal/rectal, or in both locations. McNemar’s test was then used to determine if the proportion in either the proximal or distal/rectal location was significantly different. Pearson’s chi-squared test was used to assess whether the presence of at least one of the polyp of interest was significantly associated with gender. Clustering on the presence and absence of each polyp per person (excluding serrated polyp unclassified and goblet cell hyperplastic polyps) was performed using Jaccard’s distance metric, and Ward’s clustering method. Diagnostic consistency and reproducibility was assessed using Fleiss’s kappa and intraclass correlation. The clustering was implemented using \textit{hclust} in the statistical package R. All other analysis was performed in SPSS v. 19.

RESULTS

Histological, Demographic and Anatomic Distribution Data of Colonic Polyps
During the three-month study period, 6340 polyps were received from 3603 patients (mean 1.76 polyps per patient, range 1-11). 1879 (52.2%) patients were males. The mean age for males was 60.7 years (standard deviation 13.1 years) and for females was 60.2 years (standard deviation 14.3 years). The number, location and size of each polyp type are provided in table 2. Demographic data is provided in table 3.

Conventional adenomatous polyps represented 48.7% of all colorectal polyps. The majority (86.4%) were TAs. TAs occurred more often in the proximal large bowel (58.9% proximal vs. 39.7% distal/rectal, 1.5% site not specified), however the majority of tubulovillous and villous adenomas occurred in the distal large bowel (43.7% proximal vs. 55.3% distal/rectal, 1.0% site not specified)

Using the WHO criteria, hyperplastic polyps represented 34.2% of all polyps. MVHPs accounted for 1343 of these and goblet cell hyperplastic polyps (GCHP) 825. The majority of hyperplastic polyps were distal/rectal (15.3% proximal vs. 83.5% distal, 1.2% site not specified).

Using the WHO criteria, SSAs accounted for 12.1% of all colorectal polyps and 16.6% of patients had at least one SSA. SSAs were more common in females. The mean age of
patients with at least one SSA (but no SSADs) was significantly younger than for patients with at least one SSAD (but no SSAs) (58.5 years vs. 68.9 years; p=0.0025). Of note, there was no trend for SSADs to be larger than SSAs that contrasts with adenomatous polyps where advanced features are associated with larger size (table 2).

Subcategories of MVHP and SSA with Associated Clinical Features
2084 polyps fell into the categories of MVHP, pSSA or SSA. With MVHP accounting for 1180 (56.6%), pSSA 163 (7.8%) and SSA 741 (35.6%). On a per patient basis the MVHPs were more often distal/rectal (p=<0.001) and showed no significant association with gender (tables 4 and 5). In contrast, pSSA type 1 and 2 showed no statistically significant association with location and the pSSA type 3 were more often proximal (p=0.013). As a group, the pSSAs were more often proximal (p=0.01) and more often occurred in females (p=0.037 and tables 4 and 5 and figure 3).

Of the SSAs, 178 (23.2%) had two to four SSA-type crypts, 199 (25.9%) had five to nine SSA-type crypts, 364 (47.4%) had ten or more SSA-type crypts and 27 (3.5%) were SSADs. SSAs (types 1-3) were also more likely to be proximal and to occur in females (tables 4 and 5). The mean size of these polyps tended to increase with increasing numbers of SSA-type crypts (table 6).

Clustering Analysis
Using the Jaccard distance metric and Ward’s hierarchical clustering method the patients segregated into five clusters based on the presence or absence of each type of polyp (table 7). Cluster one represents patients with only TAs, cluster two includes patients with pSSAs, SSAs, conventional type polyps and MVHPs, cluster three is patients with only TAs and MVHPs, cluster four is patients with only TAs and TVAs and cluster five is patients with only MVHPs. Diagnostics show a significant association between gender and the clusters (table 8; p-value <0.001). Cluster one and five have gender distributions similar to the entire population, however clusters three and four under-represent females, whilst cluster two over-represents females. The age distribution is also found to be significantly different between clusters (table 9; p-value <0.001). The clustering suggests that those with only one MVHP tend to be the youngest (cluster five). Those with only TVAs, or TVAs and at least one TA tend to be the oldest (cluster four). The other clusters do not significantly differ compared to the overall average.
Diagnostic Concordance Between Pathologists for MVHP Versus SSA

Four pathologists reviewed 90 polyps of interest. The overall Fleiss’s kappa score was 0.66 indicating good concordance between pathologists (table 10). There was 100% agreement between all four pathologists in 63/90 (72%) cases, including all polyps in the SSA types 1-3 categories. At least three of the four pathologists agreed on the diagnosis of 26/30 (87%) MVHP cases and in 27/30 (90%) pSSA cases.

DISCUSSION

The diagnosis and reported prevalence of serrated colorectal polyps, in particular the SSA, continues to evolve. This is unsurprising given the increasing detection of proximal sessile polyps by gastroenterologists and the relatively recent pathological description of the SSA as a distinct entity\(^{20}\). The diagnostic criteria for any new entity are expected to be refined as the salient clinical, pathological and molecular features come to light.

The primary aim of this paper was to determine the proportion of colorectal polyps that are SSAs using the diagnostic criteria of both the 2010 WHO Classification and the recent consensus document. The rates of 12.1 and 14.7% obtained in this study are the highest in the reported literature. These results are based on central pathological review with clearly defined criteria for diagnosis. These findings have been further validated in the concordance arm of the paper and as such the results are considered robust.

In previous publications the basis for the diagnosis of a SSA has not been clearly defined. Many studies based the diagnosis on the criteria outlined by Torlakovic and Snover in their seminal description of the SSA in 2003\(^{20}\) or on the features described in the excellent review of Snover et al of 2005\(^{21}\). However, because the SSA was a new entity at the time of these publications, they could not provide strict definitions as to what constitutes a SSA-type crypt or how many SSA-type crypts are required for diagnosis. In other papers very restrictive criteria were applied and as such SSAs were diagnosed only infrequently\(^{22}\).

The 2010 WHO classification and the consensus document go some way to addressing this issue but are understandably restricted by a lack of published evidence in the area. The works of Mohammadi et al and Aust et al, also provide more didactic criteria for diagnosing a SSA\(^{13,23}\). However, to the best of our knowledge, no publication to date has strictly defined and applied in a systematic fashion, both what constitutes a SSA-type crypt and how many SSA-type crypts are required to diagnose a SSA. We believe that this lack
of strict criteria is a key factor in the marked variability of SSA diagnosis in the published literature and for the high inter-observer variability in the diagnosis of SSAs\textsuperscript{10, 24, 25}.

There are several possible reasons for our higher rate of SSA diagnosis, compared to previous studies. Firstly, we strictly applied the criteria of the WHO classification and the consensus document to make our diagnoses. In many practices, particularly outside of the USA, the criteria used prior to (and since) these publications were more stringent and resulted in fewer diagnoses of SSA (and proportionally more MVHPs)\textsuperscript{21, 22}. Applying stricter criteria to our data (accepting only SSAs with five or more SSA-type crypts) would result in the percentage of SSAs decreasing from 12.1% to 9.1%. Secondly, the gastroenterological community is becoming increasingly aware of the SSA as an entity. Endoscopically, SSAs are subtle polyps and can be easily missed. Recent publications highlighting clues to the recognition of SSAs have likely resulted in greater numbers being identified and removed at colonoscopy\textsuperscript{26-28}. Finally, there has been continual improvement in technical factors relating to colonoscopy, including but not limited to quality targets (adenoma detection rates, withdrawal times, caecal intubation rates), the optical quality of colonoscopes, the quality of bowel preparation, cap-assisted colonoscopy, narrow-band imaging and magnification chromoendoscopy\textsuperscript{29}. Although the evidence supporting some of these techniques is limited, the overall quality of colonoscopy is almost certainly improving, allowing smaller and subtler polyps to be identified and safely removed.

In contrast to our high overall rate of SSAs, we had a relatively low proportion of SSADs (3.5% of SSAs). In previous publications, SSADs accounted for between 7.0% and 17.3% of all SSAs\textsuperscript{7, 8, 16}. A likely explanation for this discrepancy is the lower rate of SSAs diagnosed in these studies, resulting in a higher relative proportion of dysplastic SSAs.

The second aim of this study was to critically assess the diagnostic criteria set out in both the WHO classification and the consensus document to test the hypothesis that less stringent criteria may be adequate to diagnose a SSA. To achieve this, all MVHPs and SSAs were divided into unique subcategories dependent on the number of SSA-type crypts per polyp and analysed on a per patient basis. We found an increase in proximal
location of serrated polyps as the number of SSA-type crypts increased (figure 3). Of particular interest is the sudden shift to distal predominance for MVHPs (p<0.001), compared with the pSSAs (types 1-3) that were found significantly more often in the proximal colon (p=0.01) (table 4 and figure 3). In addition, like the SSAs, gender was significantly associated with the presence of a pSSA (p=0.037; table 5), whereas there was no significant gender relationship for the MVHPs (p=0.209; table 5). By cluster analysis, the presence of a SSA or pSSA seems, along with the other cluster two polyps, more strongly associated with females. These data suggest that serrated polyps with as few as one abnormal SSA-type crypt are more closely related to the SSA than to the MVHP and support the position of the consensus document.

Finally, we conducted a reproducibility study to validate the proposed cut-off for the diagnosis of SSA. Reproducibility between the study pathologists was good. Discordant cases were limited to the interface between MVHP and pSSA types 1-3. A degree of discordance is inevitable when separating lesions occurring on a diagnostic continuum and our series is no exception. However, our reproducibility is higher than previously reported\(^\text{25}\) and this likely reflects the relative simplicity of both our diagnostic cut-off and our diagnostic criteria.

At a clinical level these results have significant implications, particularly to surveillance colonoscopy. At present a diagnosis of MVHP does not affect follow-up intervals. In contrast, the diagnosis of a SSA is typically followed by colonoscopic surveillance similar to that of patients with conventional adenomas\(^\text{30}\). The current guidelines for colonoscopic surveillance are based on the correct pathological diagnosis of serrated polyps following the WHO definition. Failure to adequately diagnose SSAs may contribute to the occurrence of interval carcinomas. The documented association between interval carcinomas, proximal location and the serrated neoplasia pathway has been a concern for some time\(^\text{31, 32}\). Previously the assumption has been that missed polyps, incompletely excised polyps and rapid development of de novo carcinomas are the primary reasons for these interval carcinomas\(^\text{15, 33, 34}\). These results suggest that inappropriate pathological classification, with a subsequent lack of surveillance, may be another important contributing factor.

This study has several limitations. Most obvious is the lack of data on negative colonoscopies. This study was performed in a community-based pathology practice with a
large number of referring colonoscopists and as such it was not feasible to collect data on negative colonoscopies. Thus prevalence data cannot be ascertained. Also, this study included any patient with a polyp across the three-month study period and is not limited to index colonoscopies for screening purposes. Thus the age-range and indication for colonoscopy is very broad and includes patients with inflammatory bowel disease, previous CRC and familial CRC syndromes. We elected not to exclude such cases for two reasons. Firstly, in many instances little clinical history was provided and as such it would be impossible to be certain that all relevant cases were excluded and secondly we wanted the data to reflect all material coming through a community practice on a day-to-day basis. Although reproducibility was good, this study was conducted by specialist gastrointestinal pathologists who work in the same practice. This may bias towards better concordance. A multicenter reproducibility study, including non-specialist pathologists, would be required to validate our findings. Finally, although we demonstrate that serrated polyps with as few as one SSA-type crypt share gender and location parameters with more typical SSAs, the clinical significance of these polyps is not clear. Similarly, the significance of proximal versus distal serrated polyps remains to be clarified. In particular many authorities place less significance on distal polyps, even if they have histological features of a SSA. Of note, 11% of SSADs in this series were from the distal colon, suggesting that at least some distal SSAs have malignant potential. At present however, there is insufficient data from prospective studies to adequately address these issues. Hopefully such studies will be performed in the future and will address the key features of size, location and SSA-type crypt numbers.

CONCLUSIONS
In this study, we identified two points of substantial importance to both pathologists and gastroenterologists. First the proportion of colorectal polyps that are SSAs is significantly higher than has been previously published. Second, pSSAs, representing 2.6% of all colorectal polyps, have features more in common with the SSA than the MVHP. Our findings suggest that serrated polyps with any crypts displaying abnormal SSA-like architecture should be classified as a SSA. If these polyps were included with the other frank SSAs, the proportion of SSAs is 14.7% of all colorectal polyps. Further longitudinal studies are required to validate the clinical significance of these findings for the risk of subsequent SSA and of malignancy.
REFERENCES


<table>
<thead>
<tr>
<th>Subcategory</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>MVHP</td>
<td>No SSA-type crypts</td>
</tr>
<tr>
<td>pSSA (type 1)</td>
<td>One SSA-type crypt</td>
</tr>
<tr>
<td>pSSA (type 2)</td>
<td>Two non-adjacent SSA-type crypts</td>
</tr>
<tr>
<td>pSSA (type 3)</td>
<td>Multiple crypts with poorly-developed SSA-type features</td>
</tr>
<tr>
<td>SSA (type 1)</td>
<td>Minimal WHO criteria to four SSA-type crypts</td>
</tr>
<tr>
<td>SSA (type 2)</td>
<td>Five to nine SSA-type crypts</td>
</tr>
<tr>
<td>SSA (type 3)</td>
<td>Ten or more SSA-type crypts</td>
</tr>
</tbody>
</table>

MVHP – microvesicular hyperplastic polyp; pSSA – provisional SSA; SSA – sessile serrated adenoma
<table>
<thead>
<tr>
<th>Polyp type (n=6340)</th>
<th>Total number</th>
<th>Proximal</th>
<th>Distal</th>
<th>Rectum</th>
<th>Mean size (mm) (sd)</th>
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<tbody>
<tr>
<td></td>
<td>Subtype</td>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
<td></td>
</tr>
<tr>
<td><strong>All adenomatous polyps</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tubular adenoma (LGD)</td>
<td>2648 (41.8)</td>
<td>1559 (59)</td>
<td>854 (32)</td>
<td>196 (7)</td>
<td>5.8 (3.0)</td>
</tr>
<tr>
<td>Tubular adenoma (HGD)</td>
<td>20 (0.3)</td>
<td>10 (50)</td>
<td>9 (45)</td>
<td>1 (5)</td>
<td>7.3 (4.0)</td>
</tr>
<tr>
<td>Tubulovillous adenoma (LGD)</td>
<td>363 (5.7)</td>
<td>168 (46)</td>
<td>123 (34)</td>
<td>68 (19)</td>
<td>12.9 (9.0)</td>
</tr>
<tr>
<td>Tubulovillous adenoma (HGD)</td>
<td>49 (0.8)</td>
<td>15 (31)</td>
<td>22 (45)</td>
<td>12 (24)</td>
<td>17.3 (10.1)</td>
</tr>
<tr>
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<td>6 (0.1)</td>
<td>0 (0)</td>
<td>4 (67)</td>
<td>2 (33)</td>
<td>na</td>
</tr>
<tr>
<td>Villous adenoma (HGD)</td>
<td>5 (0.1)</td>
<td>1 (20)</td>
<td>2 (40)</td>
<td>2 (40)</td>
<td>20.0 (na)</td>
</tr>
<tr>
<td><strong>All serrated polyps</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyperplastic polyp</td>
<td>GCHP 825 (13)</td>
<td>129 (16)</td>
<td>418 (51)</td>
<td>266 (32)</td>
<td>4.5 (2.4)</td>
</tr>
<tr>
<td></td>
<td>MVHP 1343 (21.2)</td>
<td>202 (15)</td>
<td>593 (44)</td>
<td>533 (40)</td>
<td>4.6 (2.4)</td>
</tr>
<tr>
<td>Sessile serrated adenoma</td>
<td>741 (11.7)</td>
<td>594 (80)</td>
<td>128 (17)</td>
<td>11 (1)</td>
<td>8.5 (4.1)</td>
</tr>
<tr>
<td>Sessile serrated adenoma with dysplasia</td>
<td>27 (0.4)</td>
<td>21 (78)</td>
<td>3 (11)</td>
<td>0 (0)</td>
<td>7.8 (3.6)</td>
</tr>
<tr>
<td>Traditional serrated adenoma</td>
<td>57 (0.9)</td>
<td>18 (32)</td>
<td>22 (39)</td>
<td>17 (30)</td>
<td>10.6 (6.8)</td>
</tr>
<tr>
<td>Serrated polyp unclassifiable</td>
<td>20 (0.3)</td>
<td>14 (70)</td>
<td>6 (30)</td>
<td>0 (0)</td>
<td>4.7 (1.5)</td>
</tr>
<tr>
<td>Malignant polyp</td>
<td>23 (0.4)</td>
<td>8 (35)</td>
<td>12 (52)</td>
<td>3 (13)</td>
<td>20 (12.7)</td>
</tr>
</tbody>
</table>

*Some percentages do not add to 100 as site data was not supplied in all cases; LGD – low grade dysplasia; HGD – high grade dysplasia*
Table 3. Incidence of polyps in the study population using WHO diagnostic criteria

<table>
<thead>
<tr>
<th>PER PATIENT (n=3603)</th>
<th>Polyp type</th>
<th>Subtype</th>
<th>Total number</th>
<th>Mean age (sd)</th>
<th>Male n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All adenomatous polyps</td>
<td>Tubular adenoma (LGD)</td>
<td></td>
<td>1908 (53.0)</td>
<td>62.3 (12.5)</td>
<td>1078 (56.5)</td>
</tr>
<tr>
<td></td>
<td>Tubular adenoma (HGD)</td>
<td></td>
<td>20 (0.6)</td>
<td>67.8 (11.0)</td>
<td>12 (60.0)</td>
</tr>
<tr>
<td></td>
<td>Tubulovillous adenoma (LGD)</td>
<td></td>
<td>318 (8.8)</td>
<td>64.0 (11.6)</td>
<td>189 (59.4)</td>
</tr>
<tr>
<td></td>
<td>Tubulovillous adenoma (HGD)</td>
<td></td>
<td>47 (1.3)</td>
<td>67.0 (12.6)</td>
<td>33 (70.2)</td>
</tr>
<tr>
<td></td>
<td>Villous adenoma (LGD)</td>
<td></td>
<td>6 (0.2)</td>
<td>61.8 (21.5)</td>
<td>1 (16.7)</td>
</tr>
<tr>
<td></td>
<td>Villous adenoma (HGD)</td>
<td></td>
<td>5 (0.1)</td>
<td>64.2 (6.1)</td>
<td>4 (80.0)</td>
</tr>
<tr>
<td>All serrated polyps</td>
<td>GCHP</td>
<td>Hyperplastic polyp</td>
<td>669 (18.6)</td>
<td>59.7 (13.0)</td>
<td>356 (53.2)</td>
</tr>
<tr>
<td></td>
<td>MVHP</td>
<td></td>
<td>1068 (29.6)</td>
<td>58.2 (14.3)</td>
<td>559 (52.3)</td>
</tr>
<tr>
<td></td>
<td>Sessile serrated adenoma</td>
<td></td>
<td>579 (16.1)</td>
<td>58.6 (15.0)</td>
<td>258 (44.6)</td>
</tr>
<tr>
<td></td>
<td>Sessile serrated adenoma with dysplasia</td>
<td></td>
<td>25 (0.7)</td>
<td>69.4 (12.6)</td>
<td>11 (44.0)</td>
</tr>
<tr>
<td></td>
<td>Traditional serrated adenoma</td>
<td></td>
<td>54 (1.5)</td>
<td>61.9 (13.4)</td>
<td>27 (50.0)</td>
</tr>
<tr>
<td></td>
<td>Serrated polyp unclassifiable</td>
<td></td>
<td>19 (0.5)</td>
<td>63.4 (15.0)</td>
<td>8 (42.1)</td>
</tr>
<tr>
<td>Malignant polyp</td>
<td></td>
<td></td>
<td>23 (0.6)</td>
<td>66.5 (14.4)</td>
<td>9 (39.1)</td>
</tr>
</tbody>
</table>

LGD – low grade dysplasia; HGD – high grade dysplasia
Table 4. Location of serrated polyps on a per patient basis, sub-categorised by sessile serrated adenoma-type crypts.

<table>
<thead>
<tr>
<th></th>
<th>Proximal</th>
<th>Distal/Rectal</th>
<th>Both</th>
<th>Total</th>
<th>McNemar's Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>MVHP</td>
<td>67 (7)</td>
<td>854 (90)</td>
<td>27 (3)</td>
<td>948</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>pSSA (type 1)</td>
<td>17 (53)</td>
<td>15 (47)</td>
<td>0 (0)</td>
<td>32</td>
<td>0.86</td>
</tr>
<tr>
<td>pSSA (type 2)</td>
<td>22 (61)</td>
<td>14 (39)</td>
<td>0 (0)</td>
<td>36</td>
<td>0.24</td>
</tr>
<tr>
<td>pSSA (type 3)</td>
<td>55 (63)</td>
<td>31 (36)</td>
<td>1 (1)</td>
<td>87</td>
<td>0.013</td>
</tr>
<tr>
<td>pSSA (types 1-3)</td>
<td>92 (60)</td>
<td>59 (39)</td>
<td>2 (1)</td>
<td>153</td>
<td>0.01</td>
</tr>
<tr>
<td>SSA (type 1)</td>
<td>121 (74)</td>
<td>38 (23)</td>
<td>5 (3)</td>
<td>164</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SSA (type 2)</td>
<td>145 (81)</td>
<td>32 (18)</td>
<td>3 (2)</td>
<td>180</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SSA (type 3)</td>
<td>245 (82)</td>
<td>42 (14)</td>
<td>13 (4)</td>
<td>300</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

MVHP – microvesicular hyperplastic polyp; pSSA – provisional SSA; SSA – sessile serrated adenoma
Table 5. Gender of serrated polyps sub-categorised by sessile serrated adenoma-type crypts.

<table>
<thead>
<tr>
<th></th>
<th>F</th>
<th>M</th>
<th>Chi-squared test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Has MVHP</td>
<td>443 (46%)</td>
<td>516 (54%)</td>
<td>0.209</td>
</tr>
<tr>
<td>Has pSSA (type 1-3)</td>
<td>87 (56%)</td>
<td>68 (44%)</td>
<td>0.037</td>
</tr>
<tr>
<td>Has SSA (type 1-3)</td>
<td>321 (55%)</td>
<td>258 (45%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Has SSA (type 1-3)/pSSA (type 1-3)</td>
<td>399 (56%)</td>
<td>315 (44%)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

MVHP – microvesicular hyperplastic polyp; pSSA – provisional SSA; SSA – sessile serrated adenoma
Table 6. Average sizes for MVHPs and SSAs.

<table>
<thead>
<tr>
<th>Crypts</th>
<th>Mean</th>
<th>N</th>
<th>Std. Deviation</th>
</tr>
</thead>
<tbody>
<tr>
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<td>4.5</td>
<td>224</td>
<td>2.1</td>
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<tr>
<td>pSSA (type 1)</td>
<td>5.8</td>
<td>5</td>
<td>2.5</td>
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<tr>
<td>pSSA (type 2)</td>
<td>7.1</td>
<td>9</td>
<td>5.1</td>
</tr>
<tr>
<td>pSSA (type 3)</td>
<td>5.4</td>
<td>15</td>
<td>2.3</td>
</tr>
<tr>
<td>SSA (type 1)</td>
<td>7.5</td>
<td>38</td>
<td>3.4</td>
</tr>
<tr>
<td>SSA (type 2)</td>
<td>7.6</td>
<td>47</td>
<td>3.3</td>
</tr>
<tr>
<td>SSA (type 3)</td>
<td>9.6</td>
<td>67</td>
<td>4.7</td>
</tr>
</tbody>
</table>

MVHP – microvesicular hyperplastic polyp; pSSA – provisional SSA; SSA – sessile serrated adenoma
Table 7. Patients segregated using the Jaccard distance metric and Ward’s hierarchical clustering method.

<table>
<thead>
<tr>
<th>Cluster</th>
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<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<tr>
<td>TA</td>
<td>1314</td>
<td>281</td>
<td>216</td>
<td>113</td>
<td>0</td>
</tr>
<tr>
<td>TVA</td>
<td>0</td>
<td>84</td>
<td>0</td>
<td>270</td>
<td>0</td>
</tr>
<tr>
<td>SSAD</td>
<td>0</td>
<td>25</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>VA</td>
<td>0</td>
<td>11</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TSA</td>
<td>0</td>
<td>54</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MVHP</td>
<td>0</td>
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<td>216</td>
<td>0</td>
<td>512</td>
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<td>pSSA (type 1-3)</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SSA (type 1)</td>
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<td>165</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SSA (type 2)</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
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<td>SSA (type 3)</td>
<td>0</td>
<td>303</td>
<td>0</td>
<td>0</td>
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</table>

TA – tubular adenoma; TVA – tubulovillous adenoma; SSAD – sessile serrated adenoma with dysplasia; VA – villous adenoma; TSA – traditional serrated adenoma; MVHP – microvesicular hyperplastic polyp; pSSA – provisional SSA; SSA – sessile serrated adenoma
Table 8. Gender distribution amongst the clusters.

<table>
<thead>
<tr>
<th>Gender</th>
<th>Cluster 1</th>
<th>Cluster 2</th>
<th>Cluster 3</th>
<th>Cluster 4</th>
<th>Cluster 5</th>
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<tr>
<td>F</td>
<td>590 (45%)</td>
<td>448 (55%)</td>
<td>81 (38%)</td>
<td>107 (40%)</td>
<td>245 (48%)</td>
</tr>
<tr>
<td>M</td>
<td>725 (55%)</td>
<td>370 (45%)</td>
<td>135 (62%)</td>
<td>162 (60%)</td>
<td>267 (52%)</td>
</tr>
</tbody>
</table>

Pearson’s Chi-squared test statistic reveals a significant difference between the groups (p<0.001)
Table 9. Age distribution amongst the clusters.

<table>
<thead>
<tr>
<th></th>
<th>Mean age</th>
<th>Std. Dev</th>
<th>95 % CI LB</th>
<th>95 % CI UB</th>
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<td>62.3</td>
<td>12.3</td>
<td>61.6</td>
<td>63.0</td>
</tr>
<tr>
<td>2</td>
<td>59.1</td>
<td>14.7</td>
<td>58.1</td>
<td>60.1</td>
</tr>
<tr>
<td>3</td>
<td>61.0</td>
<td>13.4</td>
<td>59.2</td>
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<tr>
<td>4</td>
<td>64.5</td>
<td>12.1</td>
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<tr>
<td>5</td>
<td>57.3</td>
<td>14.4</td>
<td>56.0</td>
<td>58.5</td>
</tr>
<tr>
<td>Total</td>
<td>60.7</td>
<td>13.6</td>
<td>60.3</td>
<td>61.2</td>
</tr>
</tbody>
</table>

One-way ANOVA indicates there is a significant difference between the groups \( p<0.001 \); LB – lower boundary; UB – upper boundary
Table 10. Overall interobserver agreement between pathologists (30 MVHP, 30 pSSA (type 1-3) and 30 SSA (type 1-3))

<table>
<thead>
<tr>
<th>Statistic</th>
<th>Statistic</th>
<th>p-value</th>
<th>Interobserver agreement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intraclass Correlation</td>
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<td></td>
<td></td>
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<tr>
<td>- Agreement</td>
<td>0.89 (0.85 -0.92)</td>
<td>&lt;0.01</td>
<td></td>
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<tr>
<td>- Consistency</td>
<td>0.67 (0.58 -0.75)</td>
<td>&lt;0.01</td>
<td></td>
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<td>Fleiss's kappa</td>
<td>0.66</td>
<td>&lt;0.01</td>
<td>Good</td>
</tr>
</tbody>
</table>

MVHP – microvesicular hyperplastic polyp; pSSA – provisional sessile serrated adenoma; SSA – sessile serrated adenoma
FIGURE LEGENDS

Figure 1. Features of a sessile serrated adenoma-type crypt. Horizontal growth along the muscularis mucosa, deep serration and asymmetric proliferation (A), dilation of the crypt bases (B). Haematoxylin and eosin stain.

Figure 2. Examples of study microvesicular hyperplastic polyp (A) and provisional sessile serrated adenomas type 1-3 (B-D). Haematoxylin and eosin stain.

Figure 3. Location of subcategories of microvesicular hyperplastic polyp, provisional sessile serrated adenomas and sessile serrated adenomas by percentage on a per polyp basis.
Chapter 3: A clinicopathological and molecular analysis of 200 traditional serrated adenomas

As published in Modern Pathology as:


Relevance to aims of the thesis:

This chapter addresses parts of aims 3, 4 and 6. The traditional serrated adenoma is a rare colorectal polyp.\(^1\)-\(^4\) The clinicopathological and molecular features have been assessed in several papers; however these studies have suffered from low numbers, somewhat limited investigations and possibly contamination by other polyp subtypes (in particular tubulovillous adenomas).\(^4\)-\(^7\) As a result the data in the literature is often contradictory. The distribution and origins of traditional serrated adenomas are quite variable and the proportions with \textit{BRAF} or \textit{KRAS} mutation and the CpG island methylator phenotype are not clear.\(^5\),\(^6\),\(^8\),\(^9\) Perhaps more importantly, the molecular events leading to malignant transformation have only been partially addressed.\(^5\),\(^10\) In this chapter we gathered a series of 200 traditional serrated adenomas for detailed analysis. The large size of the cohort, the strict inclusion criteria and the thorough molecular assessment has allowed a thorough assessment of the clinicopathological and molecular features of these polyps and has provided insights into the molecular subtypes of colorectal carcinoma that arise from traditional serrated adenomas.

10. Tsai JH, Liau JY, Lin YL, et al. Traditional serrated adenoma has two pathways of neoplastic progression that are distinct from the sessile serrated pathway of colorectal carcinogenesis. Mod Pathol. 2014.
A clinicopathological and molecular analysis of 200 traditional serrated adenomas

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Running Title: The traditional serrated adenoma
Abstract
The traditional serrated adenoma is the least common colorectal serrated polyp. The clinicopathological features and molecular drivers of these polyps require further investigation. We have prospectively collected a cohort of 200 ordinary and advanced traditional serrated adenomas and performed BRAF and KRAS mutational profiling, CpG island methylator phenotype analysis and immunohistochemistry for a panel of seven antibodies (MLH1, β-catenin, p53, p16, Ki67, CK7 and CK20) on all cases. The mean age of the patients was 64 years and 50% were female. 71% of polyps were distal. Advanced histology (overt dysplasia or carcinoma) was present in 19% of cases. BRAF mutation was present in 67% and KRAS mutation in 22%. BRAF mutant traditional serrated adenomas were more frequently proximal (39% versus 2%; p=<0.0001), were exclusively associated with a precursor polyp (57% versus 0%; p=<0.0001) and were more frequently CpG island methylator phenotype high (60% versus 16%; p=<0.0001) than KRAS mutant traditional serrated adenomas. Advanced traditional serrated adenomas retained MLH1 expression in 97%, showed strong p53 staining in 55% and nuclear β-catenin staining in 40%. P16 staining was lost in the advanced areas of 55% of BRAF mutant traditional serrated adenomas compared to 10% of the advanced areas of KRAS mutant or BRAF/KRAS wild type traditional serrated adenomas. BRAF and KRAS mutant traditional serrated adenomas are morphologically related but biologically disparate polyps with distinctive clinicopathological and molecular features. The overwhelming majority of traditional serrated adenomas retain mismatch repair enzyme function indicating a microsatellite stable phenotype. Malignant progression occurs via TP53 mutation and Wnt pathway activation regardless of mutation status. However, CDKN2A (encoding the p16 protein) is silenced nearly exclusively in the advanced areas of the BRAF mutant traditional serrated adenomas. Thus the BRAF mutant traditional serrated adenoma represents an important precursor of the aggressive BRAF mutant, microsatellite stable subtype of colorectal carcinoma.

Key words: Traditional serrated adenoma; serrated neoplasia pathway, colorectal carcinoma, colorectal polyps, BRAF, KRAS, CpG island methylator phenotype
Introduction

The serrated neoplasia pathway accounts for 15 to 35% of colorectal carcinoma.\(^{(1-3)}\) Well established molecular drivers of this pathway are MAP kinase pathway activation, a critical early event resulting from either activating *BRAF* or *KRAS* mutation\(^{(4)}\) and the CpG island methylator phenotype, a co-ordinate methylation of CpG islands in the promoter regions of many genes that results in gene silencing.\(^{(4-6)}\) The CpG island methylator phenotype is particularly relevant to carcinogenesis when affecting tumour suppressor genes.\(^{(5)}\) *MLH1* is the best known of these, with silencing leading to microsatellite instability. This is frequently observed in the malignant transformation of sessile serrated adenomas. However, *MLH1* methylation and microsatellite instability are not pre-requisites of serrated neoplasia.

Traditional serrated adenomas remain the least understood of the serrated polyps, probably reflecting their rarity, accounting for less than 1% of colorectal polyps in most series.\(^{(7-10)}\) They were first defined by Longacre et al\(^{(7)}\) as serrated adenomas, describing polyps with mixed hyperplastic and adenomatous features, a subset of which showed what is now considered the ‘typical cytology’ of a traditional serrated adenoma, namely cells with abundant eosinophilic cytoplasm and centrally placed pencillate nuclei.\(^{(7)}\) Since then, a diverse range of polyps including sessile serrated adenoma, sessile serrated adenoma with dysplasia and tubulovillous adenoma with prominent serration have been misclassified as traditional serrated adenomas.\(^{(11)}\) Publications by Torlakovic et al\(^{(12, 13)}\) in 2003 and 2008 improved diagnostic reproducibility by characterizing the sessile serrated adenoma and identifying key features of the traditional serrated adenoma, in particular ectopic crypt formations. The 4th edition of the WHO Classification of Tumours of the Digestive Tract emphasizes protuberant and villiform growth patterns and ectopic crypt formations in the diagnosis of the traditional serrated adenoma.\(^{(11)}\) Typical cytology is recognised as a frequent but not requisite feature.

In our experience, polyps with flat growth and absent ectopic crypt formations, but with classical traditional serrated adenoma cytology and slit-like serration are relatively common but inconsistently classified. Many pathologists consider the typical eosinophilic cell of the traditional serrated adenoma to be inherently dysplastic, yet these cells are not morphologically atypical, do not display mitotic activity and show absent or minimal proliferative activity by Ki67 staining.\(^{(11, 13)}\) Moreover, a subset of traditional serrated adenomas develop discrete areas of morphologically overt dysplasia.\(^{(14-16)}\) How to classify
these advanced traditional serrated adenomas and how to separate them from ordinary traditional serrated adenomas in routine practice has been inadequately addressed. The origin of the traditional serrated adenomas is also unclear. While some probably arise de novo, many appear to arise in a precursor polyp, especially microvesicular hyperplastic polyps or sessile serrated adenomas.\textsuperscript{(13, 16-18)}

The traditional serrated adenoma also shows more molecular heterogeneity than most other polyps. The frequency of \textit{BRAF} and \textit{KRAS} mutation and CpG island methylator phenotype-high versus CpG island methylator phenotype-low or negative is variable in the literature, which is unusual for a polyp that, above issues aside, is morphologically fairly uniform.\textsuperscript{(14, 16, 17, 19, 20)} The reasons for, and the significance of, this heterogeneity have not been investigated. Finally, the pathways by which traditional serrated adenomas progress to carcinoma have not been extensively studied.

Herein, we provide a detailed clinicopathological, morphological and molecular examination of a series of 200 traditional serrated adenomas. We aimed to address the above morphological issues and to interrogate the molecular features of these polyps, with a two-part focus on 1) \textit{BRAF} versus \textit{KRAS} mutant traditional serrated adenomas and 2) ordinary versus advanced traditional serrated adenomas. We find that traditional serrated adenomas can be flat and frequently arise in a precursor microvesicular hyperplastic polyp or sessile serrated adenoma. Furthermore, although \textit{BRAF} and \textit{KRAS} mutant traditional serrated adenomas are morphologically related, they are biologically distinct polyps, with differing clinicopathological and molecular features that culminate in different subtypes of colorectal carcinoma.

\textbf{Materials and Methods}

\textbf{Patients and Samples}
Two hundred traditional serrated adenomas from 196 patients were included in the study. Cases were prospectively collected between June 2007 and June 2013 during routine reporting by one author (NW) at Envoi Specialist Pathologists. All cases were reviewed by two pathologists (MB, NW) and included only if both pathologists were in agreement on the diagnosis. The series included traditional serrated adenomas removed by polypectomy, endoscopic mucosal resection, transanal endoscopic micro-surgery and colectomy. Cases from patients with known inflammatory bowel disease or a polyposis syndrome
were excluded. Clinicopathological data including patient age, gender, polyp size and location were collected from a combination of the pathology request form, specimen container and pathology report. In this study proximal includes caecum, ascending colon, hepatic flexure and transverse colon; distal includes splenic flexure, descending colon, sigmoid colon and rectum. Fifty tubulovillous adenomas without morphological evidence of serration were collected as a control group. Tubulovillous adenomas were selected as the control group as they more closely simulate the morphology of the traditional serrated adenoma than do tubular adenomas. The study was approved by the ethics committee of QIMR Berghofer Medical Research Institute (P1298).

**Histopathological Inclusion Criteria**

Inclusion criteria were based on previously published features.\(^{(3, 7, 11-13)}\) All polyps displayed at least two of the following three features; 1) typical cytology 2) slit-like epithelial serrations and 3) ectopic crypt formations; with at least one feature evident in >50% of the polyp (Figure 1a-b).

Typical cytology referred to cells with abundant brightly eosinophilic cytoplasm with centrally placed, pencillate nuclei\(^{(7)}\) (Figure 1b); slit-like epithelial serrations referred to narrow slits in the epithelium similar to normal small intestinal mucosa\(^{(3)}\) (Figure 1b) and ectopic crypt formations referred to epithelial buds with their bases not seated adjacent to the muscularis mucosae (Figure 1b).\(^{(13)}\)

**Diagnosis and validation of flat growth pattern**

Each polyp was assessed for growth pattern (flat versus protuberant). A flat growth pattern was diagnosed when the majority of the polyp was elevated less than twice the height of the normal mucosa and lacked prominent viliform projections (Figure 1c-f).

Because flat growth in a traditional serrated adenoma is controversial, we further validated the diagnostic reproducibility of this subtype. A panel of four gastrointestinal pathologists (MB, NW, CR, IB), blinded to all clinicopathological information, independently assessed the flat traditional serrated adenomas along with 50 sessile serrated adenomas randomly selected from a previous study set.\(^{(10)}\) The diagnosis of traditional serrated adenoma was based on the criteria outlined above and the diagnosis of sessile serrated adenoma was based on previously published criteria.\(^{(10, 21)}\)
Identification of a precursor component
After reaching the inclusion criteria for the study, each traditional serrated adenoma initially identified as having a precursor component by the principal author (MB) was additionally assessed for the presence of a precursor polyp of any type by three additional pathologists (NW, CR, IB). The precursor component was required to represent a discrete area of the lesion with clear morphological distinction from the traditional serrated adenoma component either at the edge or underlying the traditional serrated adenoma (figure 2a-c).(16) Diagnosis of a sessile serrated adenoma precursor required the presence of at least one unequivocal sessile serrated adenoma-type crypt.(10, 21) A precursor component was diagnosed if there was consensus between all four pathologists.

Diagnosis of advanced traditional serrated adenoma
The cohort was divided into ‘ordinary’ and ‘advanced’ traditional serrated adenomas based on the presence or absence of dysplasia or carcinoma. Dysplasia required an abrupt transition from typical traditional serrated adenoma to overt cytological dysplasia (Figure 2d). Cytological features included increased nuclear size, frequent and atypical mitoses, nuclear crowding, complete loss of polarity and pseudo-stratification with nuclei extending into the upper half of the neoplastic cell.(11, 14, 15) Architectural features were crowding of glands, cribriform glands and intraluminal necrosis. Carcinoma was recognised by breach of the muscularis mucosae by cytologically dysplastic cells in concert with desmoplastic stroma.

All advanced areas were assessed for serrated morphology. In areas of dysplasia we used the criteria outlined in the WHO of cuboidal cells with eosinophilic cytoplasm, vesicular nuclei and prominent nucleoli.(11) For areas of carcinoma we used the criteria of Makinen et al(22) and required five features for a diagnosis of serrated morphology.

Immunohistochemistry
Immunohistochemistry was performed from the formalin fixed paraffin embedded blocks. Sections were cut at 4µm then dewaxed and rehydrated. Antigen retrieval for MLH1, CK7, p16 and Ki67 was performed by incubation in high pH antigen retrieval solution (pH9.0, Dako, Glostrup, Denmark) at 112°C for seven minutes. Antigen retrieval for β-catenin, p53 and CK20 was performed by incubation in low pH antigen retrieval solution (pH6.0, Biocare Medical, Concord, CA, USA) at 112°C for seven minutes.
Sections were manually stained following the manufacturers instructions. Antibodies used were: MLH1 (clone G168-15, 1:100, BD Pharmingen, Franklin Lakes, NJ, USA), β-catenin (1:600, Cell Marque, Rocklin, CA, USA), p53 (clone DO-7, 1:150, Biocare Medical, Concord, CA, USA), p16 (clone JC8, 1:150, Santa Cruz Biotechnology, Dallas, Texas, USA), Ki67 (clone MIB-1, 1:100, Dako, Glostrup, Denmark), CK7 (clone OV-TL12/30, 1:100, Dako, Glostrup, Denmark), CK20 (clone Ks20.8, 1:150, Biocare Medical, Concord, CA, USA). Slides were counterstained with Mayer’s haematoxylin.

Each marker was assessed for both intensity and extent of staining in the following compartments; basal zone, typical eosinophilic cells, ectopic crypt formations, goblet cells, dysplastic components and invasive carcinoma components. Intensity was scored as 0-3 (0=no staining, 1=weak staining, 2=moderate staining, 3=strong staining) and extent as 0-4 (0=no staining, 1=1-10% of cells, 2=11-50% of cells, 3=51-90% of cells, 4=>90% of cells). When intensity was variable, an average of the intensity was used. A final score was determined by multiplying the intensity and extent scores (minimum score=0, maximum=12).

Interpretation of each marker was as follows. Normal MLH1 expression required the presence of nuclear staining with a score of ≥3 in each compartment. Positive β-catenin required nuclear staining with a score of ≥2 in any compartment. Loss of membrane staining and cytoplasmic staining were not considered positive. Positive p53 required nuclear staining with a score of ≥6 in any compartment. Positive P16 required either cytoplasmic or nuclear staining with a score of ≥3 in any compartment. Positive Ki67 required nuclear staining with a score of ≥9 in any compartment. Positive cytokeratin 7 and cytokeratin 20 required cytoplasmic staining with a score of ≥3 in any compartment. For ordinary traditional serrated adenomas, the compartment with the highest score was used for analysis. For advanced traditional serrated adenomas, the score only in the areas of dysplasia or carcinoma was used for analysis.

**DNA Extraction**

DNA was extracted from the formalin fixed paraffin embedded blocks using the Chelex-100 extraction method (Bio-Rad Laboratories, Hercules, CA, USA). In brief, three 10µm sections were cut from the FFPE blocks and heated to 90°C in 200uL of 0.5% Tween-20 in 1xTE and then digested with 80mg of proteinase K at 55°C for three hours. After digestion, 200uL of 5% Chelex-100 was added to the samples and they were heated to
99°C, centrifuged then cooled on ice and the paraffin layer removed. 200uL of chloroform was added, the samples centrifuged for 15 minutes and the final product from the surface phase removed by manual pipette. DNA concentration was established by spectrophotometry (NanoDrop 2000, Thermo Scientific, Fremont, CA, USA). In cases where there was contamination of the formalin fixed paraffin embedded block by non-polypoid tissue, manual microdissection was performed using a sterile scalpel blade with a marked haematoxylin and eosin stained section as a guide.

**BRAF and KRAS mutation detection**

The **BRAF** V600E mutation was detected by allele-specific polymerase chain reaction as previously described.\(^{(25)}\) **KRAS** mutations were assessed by high-resolution melt analysis as previously described.\(^{(26)}\)

**CpG Island Methylator Phenotype Determination**

Polyp genomic DNA was treated with sodium bisulfite using the Epitect Fast Bisulfite Conversion Kit (Qiagen, Duesseldorf, Germany) according to the manufacturers instructions. CpG island methylator phenotype status was determined using the MethyLight technique as previously described by Weisenberger et al.\(^{(27)}\) The CpG island methylator phenotype panel genes consisted of **CACNA1G**, **IGF2**, **NEUROG1**, **RUNX3** and **SOCS1**. The output from this assay was percent of sample DNA methylated relative to a DNA reference sample, to give a percentage of methylated reference. Using previously published criteria,\(^{(27)}\) samples with ≥3 markers with a percentage of methylated reference of >10 were considered CpG island methylator phenotype-high, samples with one or two markers with a percentage of methylated reference >10 were considered CpG island methylator phenotype-low and samples with no markers >10 were considered CpG island methylator phenotype-negative. Completely methylated genomic DNA from pooled blood samples was used to generate a standard curve for each gene. Negative controls were run on each plate and the ALU gene was used to ensure the quality of each samples bisulfite treated DNA. As previously published, we considered samples to have failed if the ALU cycle threshold value (Ct) was >23.\(^{(28)}\) In addition we required the ALU representative calculated concentration to be >1000. The methylation status of the **MLH1** promoter region was also assessed using MethyLight using the same technique as described above. The probe and primer sequences are as described previously.\(^{(29)}\)

**Statistical Analysis**
Categorical variables were compared by Fisher's exact test and continuous variables by Student's t-test. A p-value of ≤0.05 was considered significant. Inter-rater agreements of diagnoses by different pathologists were estimated using Fleiss's kappa and intraclass correlation coefficients were also obtained. SPSS version 19, R version 3.0.2 and GraphPad Prism version 6.02 were used for statistical analyses.

Results

Clinicopathological data
The clinicopathological data are presented according to the presence or absence of advanced histology and by mutation status in tables 1 and 2. Advanced traditional serrated adenomas were larger (mean 25mm) and less often associated with a precursor polyp (13%) than ordinary traditional serrated adenomas (mean 16mm, 38% precursor polyp). Most traditional serrated adenomas had mutation of either BRAF (67%) or KRAS (22%) and these mutations were mutually exclusive (table 2). BRAF mutant traditional serrated adenomas were more often proximal (39%) and had more frequent origin in a precursor polyp (57%) than KRAS mutant traditional serrated adenomas, which were rarely proximal (2%) and never associated with a precursor polyp (table 2).

Table 3 outlines the inclusion criteria relative to polyp size. Smaller polyps were less likely to have ectopic crypt formations. Similarly, 19 of 76 (25%) flat traditional serrated adenomas did not have ectopic crypt formations; in contrast only 3 of 124 (2%) protuberant traditional serrated adenomas did not show ectopic crypt formations (p-value <0.0001). Table 4 demonstrates the relationship between polyp location, mutation status and flat morphology. BRAF mutant and proximal polyps were more likely to be flat than distal polyps. However, after accounting for anatomical location, no difference was observed by mutation status alone. Reproducibility of the diagnosis of flat traditional serrated adenomas compared to sessile serrated adenomas was excellent; with a kappa value of 0.93 (p-value <0.001) and the intraclass correlation coefficients absolute agreement was 0.981 (95% confidence interval 0.975-0.986; p-value <0.001)

All advanced areas were assessed for serrated morphology (Table 5). The majority of cases had serrated morphology. All cases with both dysplasia and invasive carcinoma showed the same morphology in both components. No significant associations could be identified between serrated morphology and molecular or immunohistochemical profile.
Mutation status and the CpG Island Methylator Phenotype

*BRAF* and *KRAS* mutation were present in 67% and 22% of polyps respectively. *BRAF* mutant, *KRAS* mutant and *BRAF/KRAS* wild type polyps were CpG island methylator phenotype high in 60%, 16% and 17% respectively, low in 28%, 44% and 48% respectively and negative in 11%, 40% and 35% respectively. The control tubulovillous adenomas were CpG island methylator phenotype high, low and negative in 0%, 6% and 94% respectively. *BRAF* mutation was more frequent than *KRAS* mutation (p-value <0.0001) and was more often associated with CpG island methylator phenotype-high than *KRAS* mutation (p-value <0.0001), however, *KRAS* mutant traditional serrated adenomas were more frequently CpG island methylator phenotype-high than control tubulovillous adenomas (p-value 0.0034). 75% (44/59) of proximal traditional serrated adenomas were CpG island methylator phenotype-high, compared to 35% (48/139) of distal traditional serrated adenomas (p-value <0.0001) and in addition 83% (43/59) of proximal *BRAF* mutant traditional serrated adenomas were CpG island methylator phenotype-high, compared to 46% (38/82) of distal *BRAF* mutant traditional serrated adenomas (p-value <0.0001). Despite this, distal *BRAF* mutant traditional serrated adenomas remained more likely to be CpG island methylator phenotype-high than distal *KRAS* mutant traditional serrated adenomas (p-value 0.0025). The CpG island methylator phenotype-low status was present in 44% and 48% of *KRAS* mutant and *BRAF/KRAS* wild type traditional serrated adenomas respectively but was seen in only 6% of control tubulovillous adenomas. Advanced traditional serrated adenomas showed no statistically significant association with mutation status or CpG island methylator phenotype when compared to ordinary traditional serrated adenomas. No *BRAF* mutations were identified in the control tubulovillous adenomas.

*MLH1* promoter methylation was present in 7% (9/134) of the *BRAF* mutant traditional serrated adenomas (eight ordinary and one advanced), but in none of the *KRAS* mutant or *BRAF/KRAS* wild type traditional serrated adenomas. Only the single advanced traditional serrated adenoma with *MLH1* methylation showed concordant loss of MLH1 expression by immunohistochemistry.

**Immunohistochemistry**

Table 6 outlines the immunohistochemical features of the study polyps. Amongst the ordinary traditional serrated adenomas, *BRAF* mutant traditional serrated adenomas were
more likely than KRAS mutant traditional serrated adenomas to have a high Ki67 proliferative index in the basal compartment. No other statistically significant differences in staining patterns were identified between the ordinary traditional serrated adenomas when stratified according to mutation status. Advanced traditional serrated adenomas showed significantly increased nuclear staining for β-catenin (Figure 3a and b) and p53 (Figure 3c) compared to the ordinary traditional serrated adenomas. MLH1 nuclear staining was retained in all but one case. P16 staining was lost in 42% of the dysplastic components and 89% of the invasive components in advanced BRAF mutant polyps (Figure 3d). In contrast, p16 staining was lost in only 8% of dysplastic and 17% of invasive components in the KRAS mutant and BRAF/KRAS wild type polyps.

Discussion
This clinicopathological, morphological and molecular appraisal of a large series of rigorously categorised traditional serrated adenomas was undertaken with the aim of clarifying areas of morphological and molecular uncertainty related to these polyps. We focused on identifying features that discriminated BRAF and KRAS mutant cases and on identifying pathways by which traditional serrated adenomas progress to carcinoma.

Due to the variability in traditional serrated adenoma morphology and its evolution as they enlarge, absolute diagnostic criteria are difficult to define, although typical cytology, ectopic crypt formations, slit-like luminal serrations and protuberant or villiform growth have proven useful. Slit-like serrations are possibly the most specific, differentiating traditional serrated adenomas from morphologically similar tubulovillous adenomas with prominent serration. To ensure all polyps in the study group were traditional serrated adenomas, we required all cases to show at least two of the first three features listed, with at least one present in 50% of the polyp. Table 3 shows the relative contribution of each of these features. Of note, smaller polyps are less likely to show ectopic crypt formations.

We specifically did not include protuberant or viliform growth as an inclusion criterion, as we regularly identify traditional serrated adenomas with a flat growth pattern. Because the concept of a flat traditional serrated adenoma is contentious, we performed a reproducibility study to determine whether we could reliably distinguish these polyps from sessile serrated adenomas. In this study we achieved an excellent level of inter-observer concordance, indicating that flat traditional serrated adenomas can be reliably distinguished from sessile serrated adenomas. Flat traditional serrated adenomas are
typically (but not exclusively) proximal, \textit{BRAF} mutant and arise in sessile serrated adenomas. While proximal polyps are more likely to be flat, this is unrelated to mutation status, with distal \textit{BRAF} and \textit{KRAS} mutant traditional serrated adenomas showing similar rates of flat and protuberant growth. This correlates with a previous study showing that distal sessile serrated adenomas can also be protuberant,\cite{31} thus flat versus protuberant morphology is probably secondary to location rather than being an intrinsic element of the traditional serrated adenoma.

Our clinicopathological data are in agreement with recent publications\cite{14-17}, although in our series, precursor polyps were limited to microvesicular hyperplastic polyps and sessile serrated adenomas and were exclusively associated with \textit{BRAF} mutation. \textit{BRAF} mutant traditional serrated adenomas were also more likely to be proximal than \textit{KRAS} or \textit{BRAF/KRAS} wild type traditional serrated adenomas. Other than the association with a precursor polyp, no differences in morphology were identified based on mutation status alone.

A critical morphological issue is the distinction between traditional serrated adenoma arising in a sessile serrated adenoma from a sessile serrated adenoma with cytological dysplasia. Based on our data, there are important biological differences between these two lesions, particularly relating to mismatch repair enzyme function, thus histological distinction is important. When the traditional serrated adenoma component is obvious, the diagnosis should not be difficult, however it is fairly common to see a small but discrete focus with traditional serrated adenoma-type cytology arising in an otherwise typical sessile serrated adenoma. In our opinion, this may represent senescent change, or possibly early traditional serrated adenoma arising in the sessile serrated adenoma. Some pathologists consider such change serrated dysplasia, warranting a diagnosis of sessile serrated adenoma with dysplasia. In an audit of our own practice, a subset of sessile serrated adenomas with dysplasia (mostly prior to 2012) showed this change (unpublished data). In our more recent opinion, rendering a diagnosis of sessile serrated adenoma with dysplasia in this situation will imply a significantly greater degree of malignant risk than is warranted. True sessile serrated adenomas with dysplasia have been demonstrated to show Wnt pathway activation\cite{23}, loss of mismatch repair function\cite{32}, \textit{CDKN2A} silencing\cite{24} and sometimes \textit{TP53} mutation\cite{33}. In contrast, from our data, ordinary traditional serrated adenomas arising in a sessile serrated adenoma do not have these advanced features. Thus, in our opinion, calling these lesions sessile serrated adenoma with dysplasia risks
diminishing the significance of true sessile serrated adenoma with dysplasia and potentially leading to erroneous conclusions regarding surveillance.

The final important morphological point is the diagnosis of dysplasia arising in a traditional serrated adenoma. In our series we found a very high rate of both serrated type dysplasia and carcinoma amongst our advanced polyps. This is in keeping with the work of Makinen et al, who defined serrated adenocarcinoma primarily from cancers arising in traditional serrated adenomas.\(^{22, 34}\) In a subsequent paper, Stefanius et al, showed that both KRAS and BRAF mutations are common in serrated adenocarcinomas.\(^{35}\) Our findings are in keeping with these earlier studies but are different to those more recently published by Tsai et al.\(^{15}\)

In addition, we are of the opinion that the typical traditional serrated adenoma cytology does not represent a serrated dysplastic change\(^{11}\). This is a controversial issue; the reasons for our opinion are as follows. Firstly, the typical traditional serrated adenoma cytology is not overtly atypical, secondly these cells do not show mitoses and have a very low proliferative index by Ki67 staining and thirdly they do not show the advanced features seen in the overtly dysplastic cells of advanced polyps; namely positive nuclear staining for β-catenin and p53 and loss of staining for p16. Instead of routinely describing dysplasia in traditional serrated adenomas, we propose that the nomenclature be brought into line with that of the sessile serrated adenoma, whereby the ordinary traditional serrated adenoma is simply designated traditional serrated adenoma, with no mention of cytological dysplasia, and advanced traditional serrated adenomas are designated traditional serrated adenoma with dysplasia or carcinoma as appropriate. Although different patterns of dysplasia occur, the key point is to identify traditional serrated adenomas with advanced biology and a higher risk of malignant progression as evidenced by the Wnt pathway activation, TP53 mutation and CDKN2A loss demonstrated in this study. Thus, similar to the sessile serrated adenoma, reporting the presence of overt dysplasia is the critical issue. At this time, assigning a grade or dividing dysplasia into serrated versus conventional types has no clinical utility and may only introduce confusion into the nomenclature.

The second major aim of this paper was to give a thorough account of the molecular features of the traditional serrated adenoma. To this end, we performed BRAF, KRAS and CpG island methylator phenotype analysis on all polyps in the series. In line with recent publications\(^{16, 17}\) we found that BRAF mutant traditional serrated adenomas represented
about two thirds of all cases and the vast majority of proximal traditional serrated adenomas. In contrast, \textit{KRAS} mutant traditional serrated adenomas were almost exclusively distal, with a particular predilection for the rectum. \textit{BRAF/KRAS} wild type cases were more closely aligned with \textit{KRAS} mutant polyps than \textit{BRAF} mutant cases and may have as yet undefined up-regulation of MAP kinase signaling.

CpG island methylator phenotype-high status, as expected, correlated strongly with both proximal location and \textit{BRAF} mutation. However, 16\% of \textit{KRAS} mutant traditional serrated adenomas were CpG island methylator phenotype-high and 44\% were CpG island methylator phenotype-low, indicating that methylation may still play an important role in the malignant progression of these polyps. Also, the level of CpG island methylator phenotype in the \textit{KRAS} mutant and \textit{BRAF/KRAS} wild type traditional serrated adenoma was significantly more than seen in the control tubulovillous adenomas.

Some authors have questioned the validity of including traditional serrated adenomas as part of the serrated neoplasia pathway; however, the combination of MAP kinase pathway activation and CpG island methylator phenotype is strong evidence to support their continued inclusion. Part of this confusion may stem from the lack of reliability of CpG island methylator phenotype data reported in the literature. Unfortunately, no uniform panel for the CpG island methylator phenotype assessment has been utilized. Also, in many studies, no controls were included or the control cases show the CpG island methylator phenotype far outside of what could reasonably be expected based on the reported histological diagnosis.\cite{15, 19, 20, 36} This calls into question the CpG island methylator phenotype results for some of these studies. In the present study we used the well-validated panel described by Weisenberger et al.\cite{27} In this series the vast majority of control tubulovillous adenomas were CpG island methylator phenotype-negative (94\%) and none were CpG island methylator phenotype-high, which is the expected outcome and provides strong support for the validity of our results.

The final aim of this study was to interrogate the pathways by which traditional serrated adenomas progress to carcinoma. We stained each polyp with seven immunohistochemical markers. The ordinary traditional serrated adenomas were remarkably uniform in their staining patterns. Ki67 and cytokeratin 20 predominantly showed the pattern described by Torlakovic et al, with a high Ki67 index in the ectopic crypt formations and basal crypts but very limited in the typical surface cells and the
opposite pattern with cytokeratin 20. The Ki67 index was high in essentially all areas of dysplasia or early carcinoma. A proportion of ordinary and advanced traditional serrated adenomas, particularly \textit{BRAF} mutant cases, showed an aberrant cytokeratin 7 and cytokeratin 20 immunophenotype. A previous study of \textit{BRAF} mutant colorectal carcinomas showed similar aberrations\cite{37}, but were most evident in the microsatellite unstable group. This knowledge can be important when working up malignancies of unknown origin, but is not informative in the diagnosis of individual polyps.

Perhaps the most important feature of the advanced traditional serrated adenomas was the almost uniform retention of staining for MLH1, replicating the findings of a recent publication restricted to advanced traditional serrated adenomas, in which no loss of mismatch repair function was observed in 60 cases.\cite{15} This indicates a crucial biological difference in the pathways by which traditional serrated adenomas and sessile serrated adenomas with dysplasia progress to carcinoma. In the majority of sessile serrated adenomas with dysplasia, \textit{MLH1} silencing is a critical step in malignant progression, resulting in microsatellite instability and is associated with an improved prognosis.\cite{38} In contrast essentially all colorectal carcinomas arising from traditional serrated adenomas are microsatellite stable.

\(\beta\)-catenin is the final transcription factor of the Wnt signaling pathway; activation results in increased transcription of a variety of proliferation promoting genes.\cite{39} In conventional chromosomal instability type colorectal carcinoma, the Wnt pathway is activated by \textit{APC} mutation. However, \textit{APC} mutation is uncommon in serrated pathway carcinomas, instead a variety of Wnt suppressors can be silenced by promoter methylation allowing activation of Wnt signaling.\cite{39} A shift from membranous to nuclear staining of \(\beta\)-catenin is indicative of canonical Wnt pathway activation irrespective of cause and is an effective surrogate for identifying Wnt signaling. Advanced areas of the traditional serrated adenomas showed a highly significant increase in nuclear \(\beta\)-catenin staining, indicating Wnt pathway activation as an important step in malignant progression. This finding is concordant with other studies of advanced traditional serrated adenomas.\cite{14, 15}

\textit{TP53} and \textit{CDKN2A} (encoding p16) are critical tumour suppressor genes with loss of function demonstrated in a wide range of malignancies. Strong nuclear staining for p53 is an effective surrogate for \textit{TP53} gene mutation and was seen in the majority of advanced traditional serrated adenomas in this series.\cite{40} In contrast, increased cytoplasmic or
nuclear p16 staining indicates increased production of functional protein. Normal colonic mucosa is p16 negative, whereas the basal crypts and the ectopic crypt formations of ordinary traditional serrated adenomas show weak and patchy p16 staining; advanced areas frequently show strong p16 expression. This incremental pattern is postulated to represent increasing efforts to control cell proliferation by up-regulation of CDKN2A expression. However, in the majority of BRAF mutant but not KRAS mutant or BRAF/KRAS wild type traditional serrated adenomas, p16 expression is abruptly lost in areas of dysplasia and/or carcinoma. This loss is attributed to methylation induced silencing of the CDKN2A gene and appears to be an important step in the development of adenocarcinoma in these polyps. TP53 mutation has been reported previously, both as a feature of BRAF mutant, microsatellite stable colorectal carcinoma and specifically in advanced traditional serrated adenomas. However, to the best of our knowledge, loss of p16 staining has not previously been reported in advanced traditional serrated adenomas. A detailed outline of the proposed molecular pathways by which traditional serrated adenomas progress to carcinoma is provided in figure 4.

This study has limitations. Firstly, the series is not consecutive, but instead includes cases that we considered classical traditional serrated adenomas. Our inclusion criteria are those we consider to be the most diagnostically specific. Admittedly, this is based on scant evidence and needs to be confirmed in a series specifically addressing diagnostic features. The 2008 paper of Torlakovic et al is the best of this kind to date, but was limited to some extent by the nature of the study polyps, in particular, a limited number of tubulovillous adenomas resulting in a greater emphasis placed on ectopic crypt formations than perhaps is warranted. Secondly, our series has a definite bias towards larger polyps because ectopic crypt formations were selected as one of our inclusion criteria and because cases were only included if a complete molecular and immunohistochemical analysis could be performed. Generally, this is more achievable in larger polyps. This may have resulted in a greater proportion of advanced traditional serrated adenomas than might be seen in a consecutive series of traditional serrated adenomas.

In our opinion, however, these weaknesses do not detract from the important conclusions of the study. Furthermore, the clinicopathological and molecular data are very similar to that presented in other recent large series. Perhaps most relevant, in a recent study from our group that included 57 consecutive traditional serrated adenomas (1% of all colorectal polyps), the mean size (11mm), mean age (62), gender distribution (50%
female) and anatomical distribution (68% distal) were similar to what we see in this series. Advanced histology was not recorded in that study for comparison. Given these similarities, we believe that the data presented here can be inferred to be a reasonable representation of traditional serrated adenomas generally.

Conclusions
In this study we have built on the current understanding of the morphology and molecular biology of the traditional serrated adenoma. The critical morphological findings are the definite occurrence of traditional serrated adenomas arising in sessile serrated adenomas and microvesicular hyperplastic polyps and the important distinction between this process and development of sessile serrated adenomas with dysplasia. In addition, overt dysplasia arising in a traditional serrated adenoma requires distinction from the senescent change seen in ordinary traditional serrated adenomas. Thus we believe that the nomenclature of the traditional serrated adenoma should be unified with that of the sessile serrated adenoma, so that the diagnostic categories are traditional serrated adenoma (including traditional serrated adenoma arising in sessile serrated adenoma or microvesicular hyperplastic polyp) and traditional serrated adenoma with dysplasia (to encompass cases with a discrete focus of overt dysplasia). Similar to the sessile serrated adenoma, this approach will simplify the nomenclature and is also more representative of the underlying biology.

At a molecular level, traditional serrated adenomas can be broadly divided into BRAF and KRAS mutant subtypes (with BRAF/KRAS wild type cases segregating better with KRAS mutant polyps). This distinction has clinicopathological and biological significance. BRAF mutant traditional serrated adenomas are more often proximal, are regularly associated with a precursor polyp and are more frequently CpG island methylator phenotype-high. Also, CDKN2A silencing appears to be critical to malignant progression of BRAF mutant traditional serrated adenomas but not KRAS or BRAF/KRAS wild type traditional serrated adenomas.

More broadly, essentially all advanced traditional serrated adenomas show retention of MLH1 staining, implying a near universal microsatellite stable phenotype. This is expected for KRAS and BRAF/KRAS wild type traditional serrated adenomas but was unexpected for BRAF mutant cases. This finding is particularly important given that the BRAF mutant, microsatellite stable subtype of colorectal carcinoma is known to be highly aggressive.
Thus \textit{BRAF} mutant traditional serrated adenomas are an important precursor of these aggressive cancers.

At present advanced traditional serrated adenomas are not formally recognised. As such the surveillance guidelines issued by the US multi-society task force for colorectal carcinoma also do not include this entity.\textsuperscript{(41)} Given the rarity of these polyps it is unlikely that high quality evidence to direct surveillance will become available; however based on our molecular results, advanced traditional serrated adenomas are potentially aggressive lesions and we believe that in the rare instances when these polyps are identified, complete resection with close surveillance is required.

\textbf{Disclosures / Conflicts of Interest}

None to declare

\textbf{Acknowledgments}

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32  Goldstein NS. Small colonic microsatellite unstable adenocarcinomas and high-grade epithelial dysplasias in sessile serrated adenoma polypectomy specimens: a study of eight cases. Am J Clin Pathol. 2006;125:132-145.


Table 1. Clinicopathological features by advanced histology

<table>
<thead>
<tr>
<th></th>
<th>All traditional serrated adenomas (n=200)</th>
<th>Ordinary traditional serrated adenomas (n=162)</th>
<th>Advanced traditional serrated adenomas (n=38)</th>
<th>P-value (ordinary versus advanced)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td>64 (27-89)</td>
<td>64 (27-89)</td>
<td>65 (27-85)</td>
<td>0.8069</td>
</tr>
<tr>
<td><strong>Female</strong></td>
<td>50%</td>
<td>51%</td>
<td>45%</td>
<td>0.5891</td>
</tr>
<tr>
<td><strong>Mean size (mm)</strong></td>
<td>16 (3-95) (median 12)</td>
<td>14 (3-95) (median 11)</td>
<td>25 (5-70) (median 21)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>Distal location</strong></td>
<td>71%</td>
<td>68%</td>
<td>82%</td>
<td>0.1153</td>
</tr>
<tr>
<td><strong>Precursor polyp</strong></td>
<td>38%</td>
<td>44%</td>
<td>13%</td>
<td>0.0003</td>
</tr>
<tr>
<td>- sessile serrated adenoma</td>
<td>31%</td>
<td>36%</td>
<td>11%</td>
<td>0.0018</td>
</tr>
<tr>
<td>- microvesicular hyperplastic polyp</td>
<td>7%</td>
<td>8%</td>
<td>3%</td>
<td>0.4769</td>
</tr>
<tr>
<td>Feature</td>
<td>BRAF mutation (n=134)</td>
<td>KRAS mutation (n=43)</td>
<td>BRAF/KRAS wild-type (n=23)</td>
<td>P-value (BRAF versus KRAS)</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>------------------------</td>
<td>-----------------------</td>
<td>-----------------------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>Age</td>
<td>64 (27-89)</td>
<td>65 (36-86)</td>
<td>62 (36-87)</td>
<td>0.8611</td>
</tr>
<tr>
<td>Female</td>
<td>49%</td>
<td>49%</td>
<td>57%</td>
<td>1.000</td>
</tr>
<tr>
<td>Mean size (mm)</td>
<td>14 (3-70) (median 12)</td>
<td>18 (3-60) (median 13)</td>
<td>20 (4-95) (median 13)</td>
<td>0.0550</td>
</tr>
<tr>
<td>Distal location</td>
<td>61%</td>
<td>98%</td>
<td>74%</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Precursor polyp</td>
<td>57%</td>
<td>0%</td>
<td>0%</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>- sessile serrated adenoma</td>
<td>46%</td>
<td>0%</td>
<td>0%</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>- microvesicular hyperplastic polyp</td>
<td>10%</td>
<td>0%</td>
<td>0%</td>
<td>0.0233</td>
</tr>
</tbody>
</table>
Table 3. Inclusion criteria by polyp size

<table>
<thead>
<tr>
<th>Size</th>
<th>Ectopic crypt formations</th>
<th>Slit-like serrations</th>
<th>Typical cytology</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Absent &lt;50% &gt;50%</td>
<td>Absent &lt;50% &gt;50%</td>
<td>Absent &lt;50% &gt;50%</td>
</tr>
<tr>
<td>&lt;10mm</td>
<td>22% 49% 29%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=65)</td>
<td>2% 45% 54% 0 5% 95%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;10mm</td>
<td>6% 41% 53%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=135)</td>
<td>2% 44% 54% 0 4% 96%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-value</td>
<td><strong>0.0028</strong> 0.288 <strong>0.001</strong> 1.0000 1.000 1.000 1.0000 1.000 1.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(&lt;10versus&gt; 5 0 0 0 0 0 0 0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flat morphology</td>
<td>All cases</td>
<td>Proximal location</td>
<td>Distal location</td>
</tr>
<tr>
<td>---------------------</td>
<td>-------------</td>
<td>-------------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>All cases</td>
<td>76/200 (38%)</td>
<td>37/59 (63%)</td>
<td>39/141 (28%)</td>
</tr>
<tr>
<td>BRAF mutant</td>
<td>57/134 (43%)</td>
<td>33/52 (64%)</td>
<td>24/82 (29%)</td>
</tr>
<tr>
<td>KRAS mutant*</td>
<td>10/41 (24%)</td>
<td>0/1 (0%)</td>
<td>10/40 25%</td>
</tr>
<tr>
<td>Wild type</td>
<td>9/23 (39%)</td>
<td>4/6 (67%)</td>
<td>5/17 (29%)</td>
</tr>
<tr>
<td>P-value (BRAF</td>
<td>0.0436</td>
<td>0.3774</td>
<td>0.6726</td>
</tr>
<tr>
<td>versus KRAS mutant)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Location was not available for two of the KRAS mutant TSAs
Table 5. Morphology of the advanced traditional serrated adenomas

<table>
<thead>
<tr>
<th></th>
<th>Conventional morphology</th>
<th>Serrated morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRAF mutant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- dysplastic component</td>
<td>4/19</td>
<td>15/19</td>
</tr>
<tr>
<td>- invasive component</td>
<td>2/9</td>
<td>7/9</td>
</tr>
<tr>
<td>KRAS mutant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- dysplastic component</td>
<td>3/7</td>
<td>4/7</td>
</tr>
<tr>
<td>- invasive component</td>
<td>0/4</td>
<td>4/4</td>
</tr>
<tr>
<td>BRAF/KRAS wild type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- dysplastic component</td>
<td>1/6</td>
<td>5/6</td>
</tr>
<tr>
<td>- invasive component</td>
<td>0/2</td>
<td>2/2</td>
</tr>
</tbody>
</table>

Note: The study cohort included 38 advanced traditional serrated adenomas, including 22 with \(BRAF\) mutation, 10 with \(KRAS\) mutation and 6 \(BRAF/KRAS\) wild type. In some polyps both dysplasia and carcinoma were present and these components were assessed separately. In all cases displaying both dysplasia and carcinoma the two components had the same morphology.
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Ordinary traditional serrated adenoma (n=162)</th>
<th>Advanced traditional serrated adenoma (n=38)</th>
<th>Tubulovillous adenoma (n=50)</th>
<th>P-value (ordinary versus advanced)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MLH1 loss</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- all polyps</td>
<td>0%</td>
<td>3%</td>
<td>0%</td>
<td>0.35</td>
</tr>
<tr>
<td>- BRAF mutant</td>
<td>0%</td>
<td>5%</td>
<td></td>
<td>0.31</td>
</tr>
<tr>
<td>- KRAS mutant</td>
<td>0%</td>
<td>0%</td>
<td></td>
<td>1.000</td>
</tr>
<tr>
<td>- BRAF/KRAS wild type</td>
<td>0%</td>
<td>0%</td>
<td></td>
<td>1.000</td>
</tr>
<tr>
<td><strong>Nuclear β-catenin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- all polyps</td>
<td>7%</td>
<td>39%</td>
<td>84%</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>- BRAF mutant</td>
<td>5%</td>
<td>32%</td>
<td></td>
<td>0.0011</td>
</tr>
<tr>
<td>- KRAS mutant</td>
<td>12%</td>
<td>60%</td>
<td></td>
<td>0.0048</td>
</tr>
<tr>
<td>- BRAF/KRAS wild type</td>
<td>6%</td>
<td>50%</td>
<td></td>
<td>0.0401</td>
</tr>
<tr>
<td><strong>P53</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- all polyps</td>
<td>6%</td>
<td>55%</td>
<td>10%</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>- BRAF mutant</td>
<td>7%</td>
<td>45%</td>
<td></td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>- KRAS mutant</td>
<td>0%</td>
<td>70%</td>
<td></td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>- BRAF/KRAS wild type</td>
<td>6%</td>
<td>67%</td>
<td></td>
<td>0.0078</td>
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<tr>
<td><strong>P16</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>- all polyps</td>
<td>73%</td>
<td>63%</td>
<td>86%</td>
<td>0.24</td>
</tr>
<tr>
<td>- BRAF mutant</td>
<td>74%</td>
<td>45%</td>
<td></td>
<td>0.0113</td>
</tr>
<tr>
<td>- KRAS mutant</td>
<td>76%</td>
<td>90%</td>
<td></td>
<td>0.6591</td>
</tr>
<tr>
<td>- BRAF/KRAS wild type</td>
<td>59%</td>
<td>83%</td>
<td></td>
<td>0.3690</td>
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<td><strong>Ki67</strong></td>
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<tr>
<td>- BRAF mutant</td>
<td>59%</td>
<td>82%</td>
<td></td>
<td>0.0056</td>
</tr>
<tr>
<td>- KRAS mutant</td>
<td>33%</td>
<td>90%</td>
<td></td>
<td>0.0027</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>-------</td>
<td>-----</td>
</tr>
<tr>
<td></td>
<td>53%</td>
<td>83%</td>
<td>0.3401</td>
<td></td>
</tr>
<tr>
<td><strong>- BRAF/KRAS wild type</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CK7*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- all polyps</td>
<td>38%</td>
<td>32%</td>
<td>16%</td>
<td>0.5758</td>
</tr>
<tr>
<td>- <strong>BRAF</strong> mutant</td>
<td>40%</td>
<td>36%</td>
<td></td>
<td>0.8147</td>
</tr>
<tr>
<td>- <strong>KRAS</strong> mutant</td>
<td>48%</td>
<td>20%</td>
<td></td>
<td>0.1529</td>
</tr>
<tr>
<td>- <strong>BRAF/KRAS</strong> wild type</td>
<td>0%</td>
<td>33%</td>
<td></td>
<td>0.0593</td>
</tr>
<tr>
<td>CK20*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- all polyps</td>
<td>98%</td>
<td>79%</td>
<td>92%</td>
<td>0.0002</td>
</tr>
<tr>
<td>- <strong>BRAF</strong> mutant</td>
<td>97%</td>
<td>73%</td>
<td></td>
<td>0.0006</td>
</tr>
<tr>
<td>- <strong>KRAS</strong> mutant</td>
<td>97%</td>
<td>90%</td>
<td></td>
<td>0.2874</td>
</tr>
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*Immunohistochemical scoring as per materials and methods*
Figure Legends

Figure 1. (a) and (b) *BRAF* mutant traditional serrated adenoma from the rectum with typical villiform projections. (b) Higher power better demonstrating typical cytology, slit-like serrations (arrowheads) and ectopic crypt formations (arrows). (c) and (d) *BRAF* mutant traditional serrated adenoma from the sigmoid colon with flat growth pattern. Sessile serrated adenoma-type crypts underlie traditional serrated adenoma with typical cytology and slit-like serrations better demonstrated at higher power in (d). (e) and (f) *KRAS* mutant traditional serrated adenoma from the rectum with flat growth pattern. (f) Higher power of (e) showing ectopic crypt formations (arrowheads) and typical cytology in the surface epithelium.

Figure 2. (a) and (b). A protuberant *BRAF* mutant traditional serrated adenoma from the sigmoid colon with adjacent sessile serrated adenoma better demonstrated at higher power in (b). (c) A small but protuberant *BRAF* mutant traditional serrated adenoma from the rectum arising from a microvesicular hyperplastic polyp. (d) An advanced *BRAF* mutant traditional serrated adenoma from the transverse colon (left) with abrupt transition (arrow) to high grade serrated dysplasia (right). This polyp also had a small focus of invasive carcinoma (not shown); however note the carcinoma within the lymphatics of the mucosa and submucosa (asterisks).

Figure 3. (a) High power of the normal β-catenin staining pattern in an ordinary traditional serrated adenoma. Note the distinct membrane staining without cytoplasmic or nuclear staining, including in the ectopic crypt formations. (b) Abnormal pattern of β-catenin in an area of carcinoma showing strong nuclear and cytoplasmic staining. (c) Traditional serrated adenoma showing strong nuclear p53 staining in the advanced component (left) and lack of staining in the ordinary component (right). (d) Traditional serrated adenoma showing positive staining for p16 in the ordinary component (left), particularly in the proliferative basal compartment and abrupt loss of staining in the advanced component (right).

Figure 4. Proposed molecular pathways of malignant progression in *BRAF* and *KRAS* mutant traditional serrated adenoma.
**BRAF mutation pathway**

- Normal mucosa
  - → BRAF / CpG island methylator phenotype-high
  - Sessile serrated adenoma
  - → MLH1 silencing
  - Sessile serrated adenoma with dysplasia
    - → Wnt pathway activation
    - → BRAF mutant microsatellite unstable carcinoma
  - Traditional serrated adenoma with dysplasia
    - → TP53 mutation, Wnt pathway activation
  - Traditional serrated adenoma with dysplasia
    - → KRAS mutant microsatellite stable carcinoma

**KRAS mutation pathway**

- Normal mucosa
  - → KRAS / CpG island methylator phenotype-low/negative
  - Traditional serrated adenoma
  - → MLH1 silencing
  - Traditional serrated adenoma with dysplasia
    - → Wnt pathway activation
    - → BRAF mutant microsatellite stable carcinoma
  - Traditional serrated adenoma with dysplasia
    - → TP53 mutation, Wnt pathway activation
  - Traditional serrated adenoma with dysplasia
    - → KRAS mutant microsatellite stable carcinoma
Chapter 4: Clinicopathological and molecular features of sessile serrated adenomas with dysplasia and carcinoma differ by mismatch repair status

Submitted

Relevance to aims of the thesis:
This chapter addresses parts of aims 3, 4 and 6. As demonstrated in chapter 2, the sessile serrated adenoma is a common polyp.1-3 It is the precursor of the majority of serrated neoplasia pathway carcinomas and may also be the precursor of a disproportionate number of interval colorectal carcinomas.4, 5 Advanced sessile serrated adenomas (sessile serrated adenomas with dysplasia and/or carcinoma) are rare polyps.2, 6 As a result we do not have a clear picture of the clinicopathological and molecular features of these polyps. In particular the mismatch repair enzyme status of advanced sessile serrated adenomas is critical to their subsequent development;7, 8 however the frequency of this occurrence is not known and the clinicopathological implications of mismatch repair deficiency versus mismatch repair proficiency have not been investigated. These are important issues, as mismatch repair proficient advanced sessile serrated adenomas give rise to an aggressive subtype of colorectal carcinoma.9, 10 A thorough understanding of the clinical, pathological and molecular features of these polyps may help to prevent these cancers.


Clinicopathological and molecular features of sessile serrated adenomas with dysplasia and carcinoma differ by mismatch repair status

Short title: The sessile serrated adenoma with dysplasia and carcinoma

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Abbreviations: SSA – sessile serrated adenoma; SSAD – sessile serrated adenoma with dysplasia; SSADC sessile serrated adenoma with dysplasia and carcinoma; SSAC – sessile serrated adenoma with carcinoma; TSA – traditional serrated adenoma; CIMP – CpG island methylator phenotype; MMRD – mismatch repair deficient; MMRP – mismatch repair proficient; MSI – microsatellite instability

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Disclosures:
**Author Contributions:** MB, NW, CR, IB, AC, BL and VW collaborated to develop the study design. MB, SP and DM performed the experimental work. MB and NW performed the histopathological analyses. MB, SP, DM and VW interpreted the data. MB wrote the manuscript. NW, CR, IB, AC, BL and VW amended the manuscript.
Abstract

**Background and Aims:** Sessile serrated adenomas (SSA) are now recognized as important precursors of colorectal carcinoma but their biology is still incompletely understood. Study of the rare SSAs “caught in the act” of malignant transformation may provide insight into this process. The aim of this study was to perform a detailed clinicopathological and molecular analysis of a large number of advanced sessile serrated adenomas defined as sessile serrated adenomas (SSA) with dysplasia and/or carcinoma and to better define the pathways by which they progress to colorectal carcinoma.

**Methods:** This study represents a prospective series of 137 advanced SSAs diagnosed at a community gastrointestinal pathology practice in Brisbane, Australia. We performed a clinicopathological and molecular assessment of all cases. Molecular features included BRAF and KRAS mutation testing, CpG island methylator phenotype (CIMP) status (IGF2, RUNX3, CACNA1G, NEUROG1, SOCS1) and immunohistochemistry for MLH1, p53, p16, β-catenin, Ki67, MGMT.

**Results:** The mean age of the study patients was 75.4 years and 60.6% were female. The median polyp size was 9mm and 86.5% occurred in the proximal colon. BRAF V600E mutation was present in 92.7% and KRAS Codons 12 and 13 mutations in 0.7%. 94.0% were CIMP-high. 74.5% of cases lost expression of MLH1 indicating mismatch repair deficiency (MMRD). MMRD cases had significantly different clinicopathological and molecular features compared to mismatch repair proficient (MMRP) cases. MMRD cases occurred at an older age (76.8 versus 71.5 years; p=0.0036), were more common in women (69.3% versus 36.1%; p=0.0007), were more often proximal (91.5% versus 71.9%; p=0.0130), were more often CIMP-high (98.0% versus 86.1%; p=0.0137) and were less likely to show staining for p53 (6.9% versus 33.3%; p=0.0003). Loss of expression of p16 and MGMT and gain of nuclear β-catenin staining were common in both groups.

**Conclusions:** Advanced SSAs are predominantly small polyps (<10mm) and are most frequently found in individuals in their eighth decade. They can be divided into two major subtypes based on their mismatch repair status. MMRD status is associated with older age, female gender and proximal location and these polyps are the likely precursors of MMRD carcinomas with good prognosis. Both subtypes are strongly associated with mutation in BRAF rather than KRAS. Activation of the WNT signaling pathway and silencing of the p16/RB tumor suppressor pathway occurs in both subtypes as they progress towards malignancy. Inactivation of the TP53 tumour suppressor is associated with the MMRP pathway, which has been shown to have a worse clinical prognosis.
Introduction

The serrated neoplasia pathway is a major contributor to colorectal carcinoma, with approximately 25% of cases arising via this route. These cancers have their origins in serrated polyps, including sessile serrated adenomas (SSA) and traditional serrated adenomas (TSA). Of these, the SSA is by far the most prevalent and accounts for the majority of serrated neoplasia pathway carcinomas.

Sessile serrated adenomas tend to be subtle polyps that can be difficult to detect colonoscopically, are frequently incompletely excised and have the hypothesized potential for rapid malignant degeneration. For the pathologist, misdiagnosis or under-diagnosis of SSA as a microvesicular hyperplastic polyp remains an issue. This combination of factors has substantial clinical implications, the most significant of which is interval carcinoma. This can occur due to missed lesions, incompletely excised lesions, rapid progression of de novo lesions or inadequate surveillance due to misdiagnosis by the pathologist. Several studies have demonstrated that serrated pathway carcinomas are over-represented amongst interval cancers, confirming that some, if not all, of these factors are contributing to this occurrence.

SSAs occur predominantly in the proximal colon and in older women. At colonoscopy they are subtle, sessile lesions with a “cloud-like” surface. They are frequently covered by a mucus cap and rimmed by bubbles and debris. The borders of the SSA can be difficult to identify. Histologically they are characterised by abnormal crypt architecture, but without overt cytological dysplasia in the early form of the lesion. Oncogenic mutation of the BRAF gene and development of the CpG island methylator phenotype (CIMP) are characteristic molecular features. Progression to the sessile serrated adenoma with dysplasia (SSAD) heralds an aggressive phase in polyp development and is accompanied by underlying molecular events. The most common of these is methylation induced silencing of the tumour suppressor gene MLH1, resulting in loss of immunohistochemical expression of the MLH1 protein and DNA mismatch repair deficiency (MMRD). Loss of mismatch repair function allows for a rapid accumulation of mutations in genes with microsatellites (particularly microsatellites composed of mono and bi-nucleotide repeats) and thus underlies microsatellite instability (MSI). The proportion of advanced SSAs that are MMRD is not clear. In the literature, based on either MLH1 loss by immunohistochemistry or by significant MLH1 promoter methylation, this ranges between 15 and 72%. However, these studies are often confounded by small size
and older methods of polyp classification. Colonoscopically, the dysplastic component of SSADs can be difficult to detect.\textsuperscript{8, 28} Although an exophytic component has been proffered as evidence of the development of dysplasia,\textsuperscript{28} particularly in large SSAs,\textsuperscript{29} this has not been confirmed in more inclusive series.

Despite these advances, much remains unknown and there has been no large study of the clinicopathological and molecular features of SSAs with a focus of dysplasia and/or carcinoma. In particular it is unclear what percentage of advanced SSAs methylate $MLH1$. This is of critical importance as this feature underscores the MSI status of the resultant carcinomas and has major implications for treatment and prognosis.\textsuperscript{30-32} $BRAF$ mutated, microsatellite unstable cancers may not respond to conventional chemotherapy but have a good prognosis, whereas $BRAF$ mutated, microsatellite stable cancers have a very poor prognosis.\textsuperscript{30, 31, 33, 34} Additionally the contribution of $p16$, WNT pathway activation and $TP53$ mutation have not been thoroughly addressed in a large series of advanced SSAs.

Herein we investigated the clinicopathological and molecular features of a series of 137 advanced SSAs, with a particular emphasis on the dichotomy between mismatch repair deficient (MMRD) and mismatch repair proficient (MMRP) cases.

**Materials and Methods**

**Case Selection and Study Design**

This study represents a prospective series collected by one of the authors (NW) during routine reporting over a period of six years at Envoi Specialist Pathologists in Brisbane, Australia. Envoi is a high-volume community gastrointestinal pathology practice, staffed by expert gastrointestinal pathologists. Referral cases were not included in the study. All potential cases underwent pathological review by two of the authors (MB and NW). For inclusion the cases were required to show 1) a component of ordinary SSA at the edge of the lesion comprising at least three crypts, one of which must show SSA-type histology\textsuperscript{9}; 2) an abrupt transition from ordinary SSA to overt cytological dysplasia or carcinoma within the one tissue fragment and 3) exclusion of cases representing TSA arising in an SSA.\textsuperscript{35} These criteria were used to ensure that the series represented a homogenous group. Criteria one was designed to guarantee origin in an SSA, criteria two to ensure that the dysplasia or carcinoma was arising in the SSA of interest rather than being from a separate conventional adenoma collected in the same specimen jar and criteria three to
exclude the recently described phenomenon of TSA arising in an SSA.\textsuperscript{35-37} TSA arising in SSA can be easily misdiagnosed as SSAD but is a separate entity with distinct clinicopathological and biological features, thus requiring exclusion from the current study.\textsuperscript{35} Cases were additionally excluded if there was insufficient material to perform the molecular and immunohistochemical analysis. The cases included lesions removed either colonoscopically or by surgical resection and included lesions clinically considered as polyps as well as overt carcinomas. Patients with serrated polyposis syndrome were not excluded.

Ethics approval was obtained from the ethics committee of the QIMR Berghofer Medical Research Institute (P1298).

Clinicopathological Data Collection
Clinical data included patient age and gender, lesion size and lesion location. Lesion location was divided into proximal (proximal to the splenic flexure) and distal (including and distal to the splenic flexure). Pathological data included the nature of the advanced component (dysplasia and/or carcinoma), the size of the advanced components and the growth pattern of the advanced component (flat versus exophytic). Carcinoma was defined as invasion into the submucosa and specifically excludes intramucosal carcinoma (which is included with the dysplastic cases). The advanced component was diagnosed as flat if it was less than twice the height of the adjacent SSA.

Immunohistochemistry
Immunohistochemistry was performed for MLH1, p53, β-catenin, p16, Ki67 and MGMT as previously described on all cases.\textsuperscript{35}

DNA Extraction
The DNA extraction was performed on three 10um sections cut from the formalin fixed paraffin embedded blocks using the Chelex method as previously described.\textsuperscript{35}

Molecular Analyses
All cases were assessed for the \textit{BRAF} V600E mutation by allelic discrimination and for \textit{KRAS} codons 12 and 13 mutations by high resolution melt analysis as previously described.\textsuperscript{35, 38}
The CIMP status of all cases was assessed using the panel of Weisenberger et al, (SOCS1, NEUROG1, RUNX3, IGF2, CACNA1G) by quantitative methylation specific PCR as previously described.\(^{38}\) \(P16\), MLH1 and MGMT were also assessed using this technique.\(^{35}\) CIMP high required a PMR of >10 in three of the five Weisenberger et al, markers. Methylation in the other assessed genes also required a PMR of >10. To ensure the validity of the results a Ct value of <23 and an Alu representative calculated concentration of >1000 was required.\(^{35, 39}\)

Statistical Analysis
Categorical variables were compared by Fisher’s exact test and continuous variables by Student’s \(t\)-test. A p-value of \(\leq 0.05\) was considered significant. SPSS version 19, R version 3.0.2 and GraphPad Prism version 6.02 were used for statistical analyses.

Results

Final Case Mix
A total of 137 advanced SSAs from 132 patients met the diagnostic inclusion criteria and had sufficient material for the immunohistochemical and molecular analyses. These included 96 SSADs, 31 SSADCs (sessile serrated adenoma with dysplasia and carcinoma) and 10 SSACs (sessile serrated adenoma with carcinoma). 129 were clinically recognised as polyps and 8 as carcinomas. 95 were removed colonoscopically and 42 by surgical resection.

Clinicopathological Data
The clinicopathological data is presented in table 1. The mean age of the patients was 75.4 years and 61% were female. 86.5% of the polyps were proximal, the median polyp size was 9mm (mean 10.7mm) and 54% of the polyps were <10mm.

By definition all cases had a component of cytological dysplasia, invasive malignancy or both. 123/129 cases clinically recognised as a polyp had a dysplastic component, with a median size of 3mm (mean 3.6mm). 33/129 cases clinically recognised as a polyp had an invasive component, with a median size of 4mm (mean 3.8mm). A protuberant growth pattern in any part of the advanced component was present in 22 (16%) of the cases; the remainder were flat. There were no significant differences in the clinicopathological features between lesions with dysplasia only and those with an invasive component.
Further data regarding the specific location within the colorectum is given in Table 2. Although both MMRD and MMRP lesions were mostly proximal, a significant minority, especially of MMRP lesions, was found in the sigmoid colon and rectum.

Immunohistochemical data
The immunohistochemical data is presented in table 3. All lesions retained a component of ordinary SSA without dysplasia and this served as a baseline against which other components could be compared. There were highly significant changes in the staining patterns between the ordinary and advanced components of the lesions for all immunohistochemical markers. This represented increased staining for β-catenin, p53 and Ki67 and loss of staining for MLH1, p16 and MGMT in the advanced components.

Molecular data
The molecular data is presented in table 4. 93% of the cases harboured a BRAF V600E mutation and 1% had a KRAS codon 12 or 13 mutation. CIMP-high was present in 93% of cases.

Comparison of MMRD to MMRP lesions
102 (74.5%) of the cases had lost staining for the mismatch repair enzyme MLH1 indicating a MMRD phenotype. When comparing MMRD to MMRP cases, the MMRD cases occurred in older patients (mean age 76.8 versus 71.4; p=0.0033), more often in females (70% versus 34%; p=0.0003) and more often in the proximal colon (91% versus 72%; p=0.0130).

Table 5 compares the pattern of immunohistochemical staining in the advanced components of the lesions divided according to MMR status. MMRD cases were less likely to show positive p53 staining than MMRP cases (7% versus 34%; p=0.0002) and were more likely to have a high proliferative index (83% versus 69%; p=0.0172). There was no significant difference in the staining patterns of β-catenin or p16 between the MMRD and MMRP cases.

MMRD cases were more likely to be CIMP-high than MMRP cases (98% versus 80%; p=0.0010) and more likely to show MLH1 methylation (94% versus 11%; p=<0.0001). MLH1 expression by immunohistochemistry correlated tightly with MLH1 methylation by
MethyLight (p<0.0001). *MGMT* methylation was not significantly different between the MMRD and MMRP groups (42% versus 29%; p=0.1663) but correlated tightly with immunohistochemical expression (p<0.0001).

**Discussion**

The SSA is the prototype polyp of the serrated neoplasia pathway and a major contributor to the burden of colorectal carcinoma. Thus a thorough understanding of the biology of these polyps is critical to improving patient management. This series has addressed important issues relating to the clinicopathological and molecular features of advanced SSAs with a particular emphasis on the important division between MMRD and MMRP cases.

This study includes only cases diagnosed using strict histological criteria after central pathological review by expert gastrointestinal pathologists. This ensures a pure cohort of advanced SSAs without contamination by other polyp types. In particular, cases of TSA arising in an SSA and admixed tubular adenoma and ordinary SSA have been carefully excluded.35 Furthermore this series has sufficient numbers of these rare polyps to identify statistically significant subgroups.

A major problem for colonoscopists is the occurrence of interval colorectal carcinoma. As discussed, the factors that may contribute to this event include missed lesions, incompletely excised lesions, rapid progression of de novo lesions and inadequate surveillance intervals due to pathological misdiagnosis.7-9 For some, if not all of these reasons, serrated pathway carcinomas are over-represented in series of interval carcinomas. In this series we have identified an additional worrying feature: advanced SSAs are predominantly small polyps (54% <10mm). Small SSAs are more difficult to detect than large SSAs, and thus are more likely to be missed at colonoscopy. This is a particular concern given the hypothesized potential for advanced SSAs to undergo rapid malignant degeneration. Furthermore some colonoscopists may assume that the dysplastic component of an SSAD is protuberant making these lesions more obvious endoscopically.29 Unfortunately this does not appear to be the case, with only 16% of the advanced components showing a protuberant growth pattern. This misconception may have developed because of the misdiagnosis of TSA arising in SSA as an SSAD, in which the TSA component will frequently display protuberant growth. In most instances the only endoscopic clue to the advanced component of an SSA will be a change in the nature of
the pit pattern, usually from a Kudo type IIa to a Kudo type III or IV. However, because
the advanced component may be very small, this may be missed.

Apart from the small size and flat nature of most of the advanced SSAs in this series,
numerous other clinicopathological features have been demonstrated. Many of these are
expected based on previous studies of either ordinary SSAs or of BRAF mutated
carcinomas. In particular, the older age and the female predominance of the patients
were confirmed and the predilection for the proximal colon was again striking.

Due to their prognostic and predictive significance, the BRAF mutation status and MMR
function of colorectal carcinomas are likely to be routinely incorporated into pathology
reports at some stage in the future. In fact, many pathology practices, including our own,
already perform reflexive mismatch repair immunohistochemistry on all new colorectal
carcinoma diagnoses. A BRAF mutation effectively confirms the serrated origin of a
colorectal carcinoma. The BRAF mutation status is also becoming increasingly relevant,
as an adverse prognostic factor. This finding is particularly powerful when combined with
the MMR status of the carcinoma. MMRD cancers have a microsatellite unstable
phenotype, which confers a good prognosis. When combined with a BRAF mutation these
cancers still tend to behave well with a reduced propensity to nodal and systemic
metastases. In contrast, BRAF mutated and MMRP (microsatellite stable) tumours are the
most aggressive molecular subtype of colorectal carcinoma.

Because of this critical dichotomy between MMRD and MMRP serrated pathway
carcinomas, these two groups were separated for the clinicopathological and molecular
analyses. Many differences were identified and these may have relevance to patient
management. At a clinicopathological level the most striking feature was the difference in
gender distribution. Seventy percent of MMRD advanced SSAs occurred in females
compared to only 34% of MMRP cases. In addition only 9% of MMRD cases arose in the
distal colorectum compared to 28% of the MMRP cases. These findings should be borne
in mind during screening/surveillance colonoscopy. In particular, SSAs in males tend to
develop into an aggressive subtype of carcinoma. Furthermore, small and distal SSAs
should not be disregarded, particularly in men. Troublingly, 13% of the MMRP advanced
SSAs arose in the rectum. By contrast, none of the MMRD cases did so.
Ordinary SSAs (i.e. SSAs without dysplasia or malignancy) were not included in this study; however previous studies have examined the clinicopathological features of these polyps.\textsuperscript{9, 43} In a recent study from our group that included 579 ordinary SSAs diagnosed using the criteria of the WHO Classification, the mean age was 58.6 years, 80% of polyps were proximal, 56% of the patients were female and the mean polyp size was 8.5mm.\textsuperscript{9} Thus it can be inferred that ordinary SSAs have a similar site and gender distribution to advanced SSAs but occur at a younger age and are slightly smaller. The lag between ordinary SSA and SSAD appears to be approximately 15 years. Importantly there is no significant difference in age of cases with dysplasia versus those with carcinoma. This provides further support to the theory that once dysplasia develops there can be a rapid progression to malignancy.

In two recent studies of colorectal carcinomas, cases were separated according to \textit{BRAF}/\textit{KRAS} mutation and mismatch repair status.\textsuperscript{41, 42} In these series the \textit{BRAF} mutated, MMRD cases occurred at a mean age of 66-67 years, in females in 69-83\% of cases and in the proximal colon in 93-95\% of cases. In contrast the \textit{BRAF} mutated, MMRP cases occurred at a mean age of 63-64 years, in females in 59-71\% of cases and in the proximal colon in 76-80\% of cases. These groups show similar overall differences between the MMRD and MMRP cases as in this study but have a younger overall age and less striking gender difference to the current series.

The molecular steps involved in the progression from ordinary SSA to cancer is an area of intensive research. \textit{BRAF} mutation is an early and likely initiating event.\textsuperscript{21, 45} Recent evidence suggests that \textit{BRAF} mutation then directs the development of CIMP.\textsuperscript{46} The occurrence of overt cytological dysplasia is usually accompanied by demonstrable molecular events. In MMRD cases, methylation induced silencing of the \textit{MLH1} gene occurs at this transition and MLH1 immunostaining is lost. In this study, \textit{MLH1} promoter methylation as measured by quantitative methylation specific PCR (MethyLight), correlated tightly with the presence or absence of immunohistochemical staining.

Besides methylation of \textit{MLH1}, other oncogenic pathways are also involved in serrated neoplasia. WNT pathway activation is present in approximately 95\% of colorectal carcinomas, although the precise role of WNT signaling in SSAs is not clear. Several studies have utilised immunohistochemistry for $\beta$-catenin to assess this issue. $\beta$-catenin is the final transcription factor of the canonical WNT signaling pathway and as such is a
useful surrogate marker for WNT pathway activation. A shift from the normal (membranous) pattern of staining to nuclear staining indicates activation of the WNT pathway. Most studies addressing the topic have demonstrated nuclear β-catenin staining in a proportion of SSADs, although the range is quite variable (50-100%). In a thorough recent paper, strong or intermediate staining was demonstrated in 60.9% of advanced SSAs (similar to our results) and correlated with methylation of the upstream WNT antagonists SFRP, MCC and AXIN2. This upstream methylation is postulated to be the mode of WNT pathway activation in the serrated neoplasia pathway. In the current series nuclear β-catenin staining was uncommon in the ordinary SSA component, consistent with previous results, and supporting the notion that WNT signaling is not a major factor in the development of SSAs as compared to conventional adenomas. However it became frequent in the dysplastic and invasive components of both the MMRD and MMRP lesions, further supporting the concept that WNT signaling plays a role in polyp progression.

Methylation induced silencing of CDKN2A (encoding p16) is also postulated to play a role in progression to malignancy in serrated pathway carcinomas. P16 is an important tumour suppressor that can induce cell cycle arrest at the G1/S checkpoint in response to uncontrolled proliferation. Immunohistochemistry for the p16 protein has been demonstrated to be an effective method to interrogate the function of this critical tumour suppressor gene. Kriegl et al, have previously demonstrated aberrant p16 staining in advanced SSAs. In that study they showed increasing p16 expression until the development of either high-grade dysplasia or invasive carcinoma, when it was suddenly lost in a definite subset of cases, presumably secondary to methylation induced silencing. We have previously demonstrated loss of p16 staining late in the malignant progression of BRAF mutated (but not KRAS mutated) TSAs. Similarly in the current study, loss of p16 staining tended to occur at a late stage in the progression of advanced SSAs, often at the histological step between high-grade dysplasia and carcinoma. There is evidence this is due to methylation induced silencing of gene expression. Despite the difference in CIMP-high status between MMRD (98%) and MMRP (80%) cases, the rates of p16 loss were almost identical between the two groups.

TP53 is a critical tumour suppressor gene. Mutation was assessed using the surrogate of p53 immunohistochemistry. Most substitution mutations of TP53 result in markedly increased nuclear expression by immunohistochemistry. In contrast, nonsense mutations
and insertions/deletions frequently result in absent expression. Overall, p53 immunohistochemistry has a specificity of 90% and sensitivity of 67% for detecting TP53 mutation. Bond et al, demonstrated significant differences in TP53 mutation rates between BRAF mutated microsatellite stable and unstable carcinomas (40.6% versus 16.9%), with the microsatellite stable group having more frequent mutation. This study is in agreement with this finding and the presence of a TP53 mutation may be part of the explanation for the poor prognosis associated with the MMRP cancers.

The role of MGMT in advanced SSAs is less clear and has not been extensively examined. In this study 28% of cases had areas with loss of staining for MGMT by immunohistochemistry. This correlated very tightly with MGMT promoter methylation by MethylLight, indicating that methylation induced silencing is a major mode of inactivation. MGMT is a DNA repair enzyme separate from the mismatch repair system. It has been correlated with KRAS mutations previously, but obviously this is not a factor in SSAs. Loss of function of MGMT may allow additional accumulation of mutations in advanced SSAs and as such may contribute to malignant progression in these polyps.

Mismatch repair proficient SSAs and advanced BRAF mutated TSAs share some molecular similarities. In a previous study of TSAs we demonstrated nuclear staining for β-catenin and p53 and loss of staining for p16 in 32%, 45% and 55% of cases respectively. This is similar to the MMRP SSAs in this study (54%, 34% and 43% respectively). Furthermore, both of these polyps give rise to the aggressive BRAF mutated microsatellite stable subtype of colorectal carcinoma. Given that the majority of BRAF mutated TSAs appear to arise in a pre-existing SSA, it is possible that the MMRP advanced SSAs have ‘skipped’ the TSA stage in their evolution but are ultimately similar polyps.

This study does have weaknesses. Firstly, some cases were excluded because of insufficient material in the blocks for complete analysis. However, this is unlikely to have adversely impacted on the key clinicopathological and molecular findings. If anything, the requirement for adequate material will have resulted in a bias towards larger polyps. Second, the cases were not microdissected for separate molecular analysis between the ordinary and advanced components of the polyps. This is unlikely to have had a material impact on the results as BRAF mutation status and CIMP are established early, although it is possible that CIMP continues to evolve as the polyps’ progress. Because
immunohistochemistry stains at a single cell level, microdissection is not required when using this technique.

In summary we have addressed the clinicopathological and molecular features of a large series of advanced SSAs with a focus on differences between MMRD and MMRP cases. We found that advanced SSAs are mostly small polyps. Although a minority, MMRP cases are the precursors of an aggressive subtype of colorectal carcinoma. They occur more often in men at a younger age than MMRD cases and a significant subset occur distally. Malignant progression of advanced SSAs occurs through a combination of *MLH1* and *CDKN2A* silencing, WNT pathway activation and *TP53* mutation. These findings may have clinical implications. At present the surveillance guidelines for SSAs, as per the US Multi Society Task Force, recommend a 5 year surveillance interval after a diagnosis of an ordinary SSA <10mm and a 3 year surveillance interval after a diagnosis of either an SSAD or an ordinary SSA >10mm. Given the demonstrable molecular events evident in SSADs and the frequent small size of these lesions, in our view, the SSAD is a higher risk lesion than a large ordinary SSA and may warrant closer surveillance.
References


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<td>76.8</td>
<td>77.5</td>
<td>75.5</td>
<td>71.4</td>
<td>71.3</td>
<td>72.0</td>
<td><strong>0.0033</strong></td>
</tr>
<tr>
<td>Gender (female)</td>
<td>83 (61%)</td>
<td>71 (70%)</td>
<td>46</td>
<td>25</td>
<td>12</td>
<td>11</td>
<td>1 (20%)</td>
<td><strong>0.0003</strong></td>
</tr>
<tr>
<td>Location*</td>
<td>109/126 (87%)</td>
<td>86/94 (91%)</td>
<td>55/58 (95%)</td>
<td>31/36 (86%)</td>
<td>23/32 (72%)</td>
<td>18/27 (67%)</td>
<td>5/5 (100%)</td>
<td><strong>0.0130</strong></td>
</tr>
<tr>
<td>Median size#</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>11</td>
<td>8.5</td>
<td>9</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Mean size#</td>
<td>10.7</td>
<td>11.1</td>
<td>11.1</td>
<td>11.1</td>
<td>9.5</td>
<td>9.3</td>
<td>11.0</td>
<td>0.1950</td>
</tr>
<tr>
<td>Size &lt;10mm#</td>
<td>70/129 (54%)</td>
<td>47/95 (49%)</td>
<td>36/66 (55%)</td>
<td>12/29 (41%)</td>
<td>22/34 (65%)</td>
<td>18/29 (62%)</td>
<td>4/5 (80%)</td>
<td>0.1668</td>
</tr>
</tbody>
</table>

*11 cases did not have location data

#excludes 8 cases presenting clinically as a carcinoma

MMRD – mismatch repair deficient; SSAD – sessile serrated adenoma with dysplasia; SSADC/C – sessile serrated adenoma with dysplasia and carcinoma or sessile serrated adenoma with carcinoma; MMRP – mismatch repair proficient;
### Table 2. Specific location of the study polyps

<table>
<thead>
<tr>
<th>Location</th>
<th>C</th>
<th>AC</th>
<th>HF</th>
<th>TC</th>
<th>SF</th>
<th>DC</th>
<th>SC</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>All (n=112)*</td>
<td>15</td>
<td>33</td>
<td>7 (6%)</td>
<td>41</td>
<td>2 (2%)</td>
<td>6 (5%)</td>
<td>4 (4%)</td>
<td>4 (4%)</td>
</tr>
<tr>
<td></td>
<td>(13%)</td>
<td>(29%)</td>
<td>(37%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMRD (n=81)*</td>
<td>14</td>
<td>23</td>
<td>5 (6%)</td>
<td>31</td>
<td>2 (2%)</td>
<td>4 (5%)</td>
<td>2 (2%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td></td>
<td>(17%)</td>
<td>(28%)</td>
<td>(38%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMRP (n=31)*</td>
<td>1 (3%)</td>
<td>10</td>
<td>2 (6%)</td>
<td>10</td>
<td>0 (0%)</td>
<td>2 (6%)</td>
<td>2 (6%)</td>
<td>4 (13%)</td>
</tr>
<tr>
<td></td>
<td>(32%)</td>
<td>(32%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C – caecum; AC – ascending colon; HF – hepatic flexure; T – transverse colon; SF – splenic flexure; D – descending colon; S – sigmoid colon; R – rectum

*25 cases did not have a specific location provided
Table 3. Immunohistochemical features of the lesions divided into ordinary and advanced components.

<table>
<thead>
<tr>
<th>Stain</th>
<th>Ordinary SSA component (n=137)</th>
<th>Advanced components (n=137)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLH1 loss</td>
<td>0 (0%)</td>
<td>102 (75%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>P16 loss</td>
<td>13 (9%)</td>
<td>59 (43%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Positive nuclear β-</td>
<td>15 (11%)</td>
<td>76 (55%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>catenin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive p53</td>
<td>0 (0%)</td>
<td>19 (14%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Positive Ki67</td>
<td>0 (0%)</td>
<td>107 (78%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>MGMT loss</td>
<td>11 (8%)</td>
<td>38 (28%)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

SSA – sessile serrated adenoma
### Table 4. Molecular features of the study lesions

<table>
<thead>
<tr>
<th>Molecular feature</th>
<th>All (n=137)</th>
<th>MMRD (n=102)</th>
<th>MMRD SSAD (n=66)</th>
<th>MMRD SSADC/SSAD (n=36)</th>
<th>MMR P (n=35)</th>
<th>MMR SSAD (n=30)</th>
<th>MMRP SSADC/SSAD (n=30)</th>
<th>P-value (MMRD versus MMRP)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BRAF mutation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>127 (93%)</td>
<td>95 (93%)</td>
<td>60 (91%)</td>
<td>35 (97%)</td>
<td>32 (91%)</td>
<td>28 (93%)</td>
<td>4 (80%)</td>
<td>0.7154</td>
</tr>
<tr>
<td><strong>KRAS mutation</strong></td>
<td>1 (1%)</td>
<td>1 (1%)</td>
<td>1 (2%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.000</td>
</tr>
<tr>
<td><strong>CIMP-H</strong></td>
<td>128 (93%)</td>
<td>100 (98%)</td>
<td>64 (97%)</td>
<td>36 (100%)</td>
<td>28 (80%)</td>
<td>24 (80%)</td>
<td>4 (80%)</td>
<td><strong>0.0010</strong></td>
</tr>
<tr>
<td><strong>MLH1 methylation</strong></td>
<td>100 (73%)</td>
<td>96 (94%)</td>
<td>61 (92%)</td>
<td>35 (97%)</td>
<td>4 (11%)</td>
<td>3 (10%)</td>
<td>1 (20%)</td>
<td>&lt;0.000</td>
</tr>
<tr>
<td><strong>MGMT methylation</strong></td>
<td>53 (39%)</td>
<td>43 (42%)</td>
<td>26 (39%)</td>
<td>17 (47%)</td>
<td>10 (29%)</td>
<td>10 (33%)</td>
<td>0</td>
<td>0.1663</td>
</tr>
</tbody>
</table>

MMRD – mismatch repair deficient; MMRP – mismatch repair proficient; SSAD – sessile serrated adenoma with dysplasia; SSADC – sessile serrated adenoma with dysplasia and carcinoma or sessile serrated adenoma with carcinoma
Table 5. Comparison of the immunohistochemical staining patterns of the MMRD versus MMRP cases

<table>
<thead>
<tr>
<th>IHC stain</th>
<th>MMRD (n=102)</th>
<th>MMRP (n=35)</th>
<th>P-value (MMRD versus MMRP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P16 loss</td>
<td>44 (43%)</td>
<td>15 (43%)</td>
<td>1.000</td>
</tr>
<tr>
<td>Positive B-catenin</td>
<td>57 (56%)</td>
<td>19 (54%)</td>
<td>1.000</td>
</tr>
<tr>
<td>Positive P53</td>
<td>7 (7%)</td>
<td>12 (34%)</td>
<td><strong>0.0002</strong></td>
</tr>
<tr>
<td>Positive Ki67</td>
<td>85 (83%)</td>
<td>22 (63%)</td>
<td><strong>0.0172</strong></td>
</tr>
<tr>
<td>Positive MGMT</td>
<td>28 (28%)</td>
<td>10 (29%)</td>
<td>1.000</td>
</tr>
</tbody>
</table>

MMRD – mismatch repair deficient; MMRP – mismatch repair proficient; IHC - immunohistochemical
Figure legend

Figure 1. (A) A low power image of a sessile serrated adenoma with dysplasia from the proximal colon, demonstrating the frequent small size and flat nature of these polyps. (B) A low power image of a larger and protuberant sessile serrated adenoma with dysplasia. This appearance is much less common.

Figure 2. A mismatch repair deficient (A) and a mismatch repair proficient (B) sessile serrated adenoma with dysplasia. Both polyps show an abrupt transition from ordinary sessile serrated adenoma to overt cytological dysplasia. (C) and (D) are the MLH1 immunohistochemical stains for each case. Note the loss of staining in (C) compared to the retained nuclear expression in (D). (E) and (F) demonstrate the p53 immunohistochemical staining patterns for the same lesions. The overexpression in (F) is indicative of TP53 mutation and highlights a major difference between mismatch repair deficient and mismatch repair proficient cases.

Figure 3. (A) A medium power image of a p16 immunohistochemical stain of a sessile serrated adenoma with dysplasia. There is markedly increased staining for p16 in the dysplastic area, presumably representing cellular efforts to prevent uncontrolled proliferation. (B) A medium power image of a different polyp containing dysplasia (right) and carcinoma (left). In this case there is abrupt loss of staining at the transition to carcinoma. (C) A medium power magnification of the same area as image (B) this time showing β-catenin staining. Scattered nuclear staining is present in the dysplasia but becomes uniform in the carcinoma, indicative of WNT pathway activation.
Chapter 5: Serrated tubulovillous adenoma of the large intestine

Under revision (minor) - Histopathology

Relevance to aims of the thesis:
This chapter addresses aim 5. At times the distinction of traditional serrated adenomas from a subset of tubulovillous adenomas can be difficult.\textsuperscript{1,2} In the current pathology literature ectopic crypt formations are particularly emphasised as a feature of traditional serrated adenomas.\textsuperscript{3} However a subset of tubulovillous adenomas have ectopic crypt formations, along with other features of serration and thus can be difficult to distinguish from traditional serrated adenomas. Thus we gathered a consecutive series of tubulovillous adenomas with prominent serration and compared these polyps to both traditional serrated adenomas and to ordinary tubulovillous adenomas. We aimed to identify histological features that could reliably separate these polyps from the control groups and to determine if they had molecular features of serrated polyps, in particular MAP kinase pathway activation and the CpG island methylator phenotype.

Serrated tubulovillous adenoma of the large intestine

Running Title: Serrated tubulovillous adenoma

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Conflicts of Interest: The authors have no conflicts of interest to declare.
Word count: 3167
ABSTRACT

Aims: Most colorectal polyps are readily classified, but a subset of tubulovillous adenomas (TVA) with prominent serrated architecture cause diagnostic confusion. We aimed to 1) identify histological features that separate serrated TVAs from both conventional TVAs and traditional serrated adenomas (TSA) and 2) perform a clinicopathological and molecular analysis to determine if the serrated TVA has unique features.

Methods: We collected 48 serrated TVAs, 50 conventional TVAs and 66 BRAF wild-type TSAs for analysis. For each polyp we performed a clinicopathological assessment, BRAF and KRAS mutation profiling, CpG island methylator phenotype status, MGMT methylation and immunohistochemical assessment of seven markers (MLH1, p16, p53, β-catenin, Ki67, CK7 and CK20).

Results: We found that serrated TVAs can be reliably diagnosed and have features distinct from both conventional TVAs and TSAs. Compared to conventional TVAs, serrated TVAs are larger, more often proximal, more histologically advanced, show more CpG island methylation and more frequent KRAS mutation. Compared to TSAs, they are more often proximal, show less CpG island methylation, more frequent MGMT methylation and more frequent nuclear staining for β-catenin.

Conclusions: The serrated TVA can be reliably diagnosed and has unique features. It represents a precursor of KRAS mutated, microsatellite stable colorectal carcinoma.

Key words: Colonic Polyp, Adenoma, Serrated, Tubulovillous Adenoma, Traditional Serrated Adenoma
INTRODUCTION

Neoplastic colorectal epithelial polyps fall into two major groups, conventional adenomas, comprising tubular adenomas (TA), tubulovillous adenomas (TVA) and villous adenomas (VA) and serrated polyps, comprising hyperplastic polyps (HP), sessile serrated adenomas (SSA) and traditional serrated adenomas (TSA). It is becoming accepted that colorectal carcinomas can be divided into 5 molecular subtypes based on $BRAF$ and $KRAS$ mutation status, CpG island methylator phenotype (CIMP) and presence or absence of microsatellite instability (MSI). These subtypes have important predictive and prognostic implications and particular polyp subtypes give rise to specific molecular subtypes of carcinoma. In general, serrated polyps give rise to $BRAF$ mutated, CIMP-high colorectal carcinomas, whereas conventional polyps give rise to $BRAF$ and $KRAS$ wild-type, CIMP-negative, microsatellite stable (MSS) carcinomas. The origins of $KRAS$ mutated carcinomas are less clear. While many undoubtedly arise from TVAs, Jass et al, reported $KRAS$ mutation as being associated with serrated architectural features.

We frequently encounter TVAs with serrated architecture, including ECFs, which give rise to serrated morphology colorectal carcinomas. These serrated tubulovillous adenomas (sTVA) have not been described in the pathology literature but appear morphologically distinct from both TSAs and conventional tubulovillous adenomas (cTVA). We anticipate that the sTVA is associated with $KRAS$ mutation and is a precursor of $KRAS$ mutated, MSS carcinomas. Furthermore, we feel that these polyps are sufficiently distinctive to allow reproducible diagnosis by pathologists.

Thus this study has a two-part focus. First we aimed to identify histological features that separated sTVAs from both cTVAs and TSAs and then performed a clinicopathological and molecular characterization of these polyps, to determine if the sTVA had unique features. Finally, we assessed the molecular pathways by which sTVAs develop into colorectal carcinoma.

MATERIALS AND METHODS

Sample selection
Four polyp cohorts were established, comprising 2 cohorts of sTVAs and 2 control cohorts. Cohort 1 comprised consecutive sTVAs (n=27) selected from a series of 412 consecutive TVAs accrued between January 30, 2012 and April 29, 2012 at Envoi Specialist Pathologists in Brisbane, Australia as part of a separate study. Cohort 2 comprised sTVAs (n=21) collected during routine sign-out at Envoi Specialist Pathologists, selected specifically for the presence of either high-grade dysplasia and/or early carcinoma and used to better define the pathways by which sTVAs progress to carcinoma but were excluded from data used to compare the polyp groups to prevent selection bias. The control cohorts comprised 50 cTVAs (cohort 3), from the same series as cohort one and 66 BRAF wild-type TSAs (cohort 4) from a separate previously published series. The control cohorts represent the polyps that are histologically most similar to the study series and those with which they are most often confused. The BRAF wild-type TSAs include cases with either a KRAS mutation (n=43) or that were BRAF/KRAS wild-type (n=23). BRAF mutated TSAs were not included as these lesions have a significantly different molecular biology to the study polyps.

The polyps were removed by any of polypectomy, endoscopic mucosal resection, transanal endoscopic microsurgery or colectomy. Proximal polyps came from sites proximal to the splenic flexure; distal polyps from sites including and distal to the splenic flexure. For the purposes of this study, advanced histology refers to either high-grade dysplasia or carcinoma. Because of the unique cytology of TSAs, advanced histology in these cases refers to the development of an area of overt high-grade cytological dysplasia or carcinoma in an otherwise typical TSA. Patients with a known history of inflammatory bowel disease or a polyposis syndrome were excluded. Patient age, gender, polyp size and anatomical location were collected from a combination of the pathology and endoscopic reports. The study was approved by the ethics committee of QIMR Berghofer Medical Research Institute (P1298).

Histopathological Inclusion Criteria

Criteria for the diagnosis of the sTVA have not been previously defined. For the purposes of this study sTVAs were diagnosed if they met all the following criteria: 1) >25% villous component, 2) morphological serration in >50% of the polyp and 3) TSA-type cytology and slit-like serrations in <10% of the polyp.
A villous structure is defined as a leaf-like or finger-like projection of epithelium overlying a small amount of lamina propria with at least a 25% component required to meet the WHO criteria for a diagnosis of TVA. Architectural serration included prominent undulation of the epithelial lining, ECFs or a maze-like growth pattern (Figures 1A-D). ECFs are increasingly recognised as not specific for the diagnosis of TSA. It is also recognised that small components of TSA type cytology (<10%) can be seen in TVAs. In comparison with sTVAs, the cTVAs did not show these features in the majority of the polyp (Figure 2A). The cytology of both the serrated and the conventional TVAs was similar, displaying overt dysplasia characterised by basally located, crowded oval nuclei, frequent mitoses and basophilic cytoplasm (Figure 2C). In contrast, the TSAs showed architectural serration and characteristic cytology, typified by cells with abundant intensely eosinophilic cytoplasm and centrally placed, palisaded, pencillate nuclei. Serrations were due to both ECFs and characteristic slit-like serrations, which refer to superficial sharp clefts in the epithelium similar to those seen in the normal small intestine (Figures 2B&D), more recently recognised as a specific feature of the TSA.

All polyps included in the study were initially selected by the principal author (MB) and then further assessed in a blinded fashion by 3 of the study pathologists (NW, IB, CR) applying the criteria outlined above. Each pathologist was required to diagnose each polyp as either a cTVA, sTVA or TSA. A Light’s kappa value was ascertained to determine diagnostic reproducibility.

After final selection of the cases an additional assessment was made for the presence of advanced histology (high-grade dysplasia and carcinoma) based on previously published criteria and required consensus between all four pathologists after review of the slides at a multi-header microscope. High-grade dysplasia and carcinoma were then separately assessed for serrated morphology, also requiring consensus between all four pathologists (Figures 3A&B).

**Immunohistochemistry**

Immunohistochemistry was performed using the formalin fixed paraffin embedded (FFPE) blocks. Four micrometer sections were cut, dewaxed and rehydrated. High pH antigen retrieval solution (pH9.0, Dako, Glostrup, Denmark) was used for MLH1, CK7, p16 and
Ki67 at 112°C for 7 minutes. Low pH antigen retrieval solution (pH6.0, Biocare Medical, Concord, CA, USA) was used for β-catenin, p53 and CK20 also at 112°C for 7 minutes.

All sections were manually stained following the manufacturers instructions. Antibodies used were: MLH1 (clone G168-15, 1:100, BD Pharmingen, Franklin Lakes, NJ, USA), β-catenin (1:600, Cell Marque, Rocklin, CA, USA), p53 (clone DO-7, 1:150, Biocare Medical, Concord, CA, USA), p16 (clone JC8, 1:150, Santa Cruz Biotechnology, Dallas, Texas, USA), Ki67 (clone MIB-1, 1:100, Dako, Glostrup, Denmark), CK7 (clone OV-TL12/30, 1:100, Dako, Glostrup, Denmark) and CK20 (clone Ks20.8, 1:150, Biocare Medical, Concord, CA, USA). Slides were counterstained with Mayer’s haematoxylin.

Each marker was assessed for intensity and extent of staining in the following compartments as appropriate to the polyp subtype; basal zone, ECFs, eosinophilic cells, low-grade dysplastic components, high-grade dysplastic components and invasive carcinoma components as previously published.9 Intensity of staining was scored as 0-3 (0=nil, 1=weak, 2=moderate, 3=strong) and extent of cells stained as 0-4 (0=nil, 1=1-10%, 2=11-50%, 3=51-90%, 4=>90%). The final score was determined by multiplying the score for intensity and extent.

Each marker was interpreted as previously published as follows.9 Abnormal MLH1 expression required absence of nuclear staining in a distinct portion of the polyp. Abnormal β-catenin expression required nuclear staining with a score of ≥2 in any compartment. Positive p53 required nuclear staining with a score of ≥6 in any compartment. Positive p16 required either cytoplasmic or nuclear staining with a score of ≥3 in any compartment. Positive Ki67 required nuclear staining with a score of ≥9 in any compartment. Positive cytokeratin 7 and cytokeratin 20 required cytoplasmic staining with a score of ≥3 in any compartment. For cases without advanced histology the compartment with the highest score was used for analysis. For cases with advanced histology, the score in the areas of high-grade dysplasia or carcinoma was used for analysis.

DNA Extraction

DNA was extracted from the FFPE blocks using the Chelex-100 extraction method (Bio-Rad Laboratories, Hercules, CA, USA) as previously described.9
BRAF and KRAS mutation detection

The BRAF V600E mutation was detected by allelic discrimination as previously described. KRAS mutations were assessed by high-resolution melt analysis as previously described.

CpG Island Methylator Phenotype and MGMT Methylation Status

CIMP status was determined using the gene panel of Weisenberger et al, (CACNA1G, IGF2, NEUROG1, RUNX3, SOCS1) as previously described. CIMP-high required >2 markers to be methylated and CIMP-low 1-2 markers methylated. MGMT methylation was determined using the MethylLight technique as previously described.

Statistical Analysis

Categorical variables were compared by Chi-square / Fisher’s exact test and continuous variables by Student’s t / Wilcoxon test. A p-value of ≤0.05 was considered significant. Inter-rater agreements of diagnoses by the study pathologists were estimated using Light’s kappa. SPSS version 19, R version 3.0.2 and GraphPad Prism version 6.02 were used for statistical analyses.

RESULTS

Clinicopathological data and diagnostic concordance

Twenty-seven polyps from the consecutive series (cohort 1) fulfilled the selection criteria for sTVA, representing 7% of all TVAs. The Light’s kappa value for agreement between the 4 study pathologists in the diagnosis of the 3 categories of polyp, was 0.85 (Bootstrap 95% confidence interval; 0.80-0.89), indicating excellent diagnostic concordance. Twenty-nine cases achieved consensus for advanced histology (8 cases from cohort 1 and all 21 cases from cohort 2), including 27 with high-grade dysplasia and 7 with invasive carcinoma (Table 1). Five cases had high-grade dysplasia and early carcinoma. Of the 27 cases with high-grade dysplasia, 2 had a conventional pattern, 12 had some serrated features but insufficient to meet the WHO criteria for serrated dysplasia and 13 had
serrated dysplasia. Of the 7 cases with invasive carcinoma, 1 had a conventional pattern and 6 had serrated morphology.

The clinicopathological data for cohorts 1, 3 and 4 are presented in table 2. The sTVAs were larger, more often proximal and more often displayed advanced histology than the cTVAs. Compared to \textit{BRAF} wild-type TSAs, sTVAs were more likely to be proximal.

\textbf{Immunohistochemistry}

The immunohistochemical profiles of the non-advanced components of cohorts 1, 3 and 4 are presented in table 3. In particular, nuclear \(\beta\)-catenin staining is more frequent in the sTVAs than the \textit{BRAF} wild-type TSAs (Figures 4A&B). The staining patterns of all of the sTVAs (cohorts 1 and 2), comparing the low-grade dysplasia components with the advanced components are presented in table 4. Nuclear p53 staining is more common in the advanced components of the sTVAs.

\textbf{BRAF and KRAS mutation profiles}

The \textit{BRAF} and \textit{KRAS} mutation status of cohorts 1, 3 and 4 are presented in table 2. No cases were \textit{BRAF} mutated. The sTVAs were more likely to harbour a \textit{KRAS} mutation than the cTVAs.

\textbf{Methylation analysis}

The CIMP status and \textit{MGMT} methylation status of cohorts 1, 3 and 4 are presented in table 2. Of note, the sTVAs are less likely to be CIMP-negative than the cTVAs but more likely to be CIMP-negative than \textit{BRAF} wild-type TSAs. The sTVAs show similar levels of \textit{MGMT} methylation to the cTVAs but more frequent methylation than \textit{BRAF} wild-type TSAs.

\textbf{DISCUSSION}

Although the TVA with architectural serration has been mentioned in textbooks and abstracts,\textsuperscript{5, 20} to the best of our knowledge there have been no previous reports in the peer-reviewed literature. Pai et al, examined the morphology of conventional adenomas
occurring in patients with synchronous serrated polyps and found several distinctive features including mild serration, cytoplasmic eosinophilia, dilated crypt bases and low-level methylation when compared to control cohorts. However, most of these polyps were TAs and none showed a KRAS mutation. Kakar et al, assessed the molecular features of a series of TAs, TVAs and VAs, not selected for serrated architecture and identified a KRAS mutation in only 9% of TVAs. More recently, Tsai et al, studied 60 TSAs with either high-grade dysplasia or carcinoma. In that cohort, 19 polyps were designated as TVA with serrated features, which appear to resemble the polyps in our series, although with more features of TSA than our cohort. Interestingly they have similar rates of KRAS mutation (79%) but have infrequent nuclear β-catenin staining (11%) and rarely give rise to serrated morphology carcinomas. Finally Hafezi-Bakhtiar et al, identified discriminating histological features useful for separating TVAs from TSAs. They found that ECFs were common in both cohorts, although more numerous in TSAs. In contrast, slit-like serrations were highly sensitive and specific for the diagnosis of TSA. Several other studies have demonstrated an association between KRAS mutation and adenoma size, villosity and high-grade dysplasia, but have not addressed the issue of serrated architecture.

In this study we established baseline histopathological features for the diagnosis of sTVAs and applied them to a consecutive series of 412 TVAs to develop a study cohort (cohort 1). The diagnostic criteria used, although to some extent arbitrary, required at least 50% histological serration to ensure that this was the dominant pattern in the study polyps.

Although sTVAs and TSAs both have architectural serration, the epithelium of sTVAs typically showed uniform conventional-type dysplasia, similar in appearance to cTVAs, but quite distinct to the eosinophilic cells of the TSA. Because many pathologists would currently diagnose these sTVAs as TSAs, the first aim of this study was to determine if they could be reproducibly diagnosed. Thus our diagnostic criteria were designed to ensure that the study polyps had 1) a prominent serrated architecture to separate them from cTVAs and 2) lacked TSA-type cytology and slit-like serrations, thus distinguishing them from TSAs. Similar to the recent study of Hafezi-Bakhtiar et al, we allowed a minor component (<10%) with TSA-type morphology, in recognition that small components of this nature are commonly present in otherwise typical serrated and conventional polyps.

Using these criteria, we found that sTVAs can be reliably diagnosed and are distinct from both TSAs and cTVAs. Compared to cTVAs, the sTVAs were larger, more often proximal
and more frequently displayed advanced histology. In addition, they more frequently harboured a \textit{KRAS} mutation and showed more frequent CIMP. Compared to \textit{BRAF} wild-type TSAs the sTVAs were more often proximal, showed less CIMP, more frequent \textit{MGMT} methylation, more frequent nuclear \(\beta\)-catenin staining and less frequent CK7 staining.

Thus \textit{BRAF} wild-type TSAs and sTVAs represent separate precursors of the \textit{KRAS} mutated, MSS subtype of colorectal carcinoma.\textsuperscript{7} The order in which polyps develop Wnt and MAP kinase pathway activation may be critical to polyp morphology and biology. The \textit{BRAF} wild-type TSAs seem to be initiated by \textit{KRAS} mutation and develop Wnt pathway activation late.\textsuperscript{9} In contrast, sTVAs develop early Wnt pathway activation as demonstrated by nuclear \(\beta\)-catenin staining (which in this context most likely reflects \textit{APC} mutation) and then develop a serrated signature, presumably as a consequence of subsequent \textit{KRAS} mutation. Thus we envisage \textit{BRAF} wild-type TSAs are more closely aligned with the serrated pathway and that the sTVAs are more closely aligned with the traditional pathway.

The second component of this study was to assess the morphology and pathways by which sTVAs progress to malignancy and to compare these with the other polyp subtypes. We found that the high-grade dysplasia and invasive carcinomas arising in sTVAs frequently demonstrated serrated morphology. Although possibly controversial, this finding is not unexpected. In his original description of serrated morphology carcinomas, Makinen assessed carcinomas arising from serrated adenomas.\textsuperscript{26} At that time serrated adenomas were not a clearly defined entity and likely included TSAs, SSAs with dysplasia and almost certainly sTVAs.\textsuperscript{1} In a subsequent work from his group, it was shown that serrated morphology carcinomas very frequently show MAP kinase pathway activation, and that this was more frequently due to \textit{KRAS} rather than \textit{BRAF} mutation.\textsuperscript{27} Thus we are not surprised to find serrated morphology in the majority of the carcinomas arising from these polyps.\textsuperscript{7}

Using immunohistochemistry, loss of expression of the mismatch repair enzyme MLH1, as expected, was not found in any sTVAs, as MSI secondary to \textit{MLH1} promoter methylation is strongly correlated with \textit{BRAF} but not \textit{KRAS} mutation.\textsuperscript{28} In contrast, strong nuclear p53 staining (indicative of \textit{TP53} mutation \textsuperscript{29}) was more frequent in the advanced components of the sTVAs. Also, sTVAs did not show loss of p16 staining, suggesting that loss of
function of CDKN2A is not important in the progression of these carcinomas. An outline of the proposed pathways to carcinoma for the various polyp types is shown in Figure 5.

In the current literature, ECFs, predominant villous architecture and distal location are emphasized in the diagnosis of TSAs. All of these features are shared with sTVAs making misdiagnosis easy. The absence of the typical TSA-type cytology and slit-like serrations is key to making the correct diagnosis. In day-to-day practice, the diagnosis of sTVAs as a distinct entity may not be necessary, as the surveillance guidelines for TVAs and TSAs are currently the same. However, it may be helpful to provide the additional information in certain scenarios. The presence of an sTVA component adjacent to an invasive carcinoma would make a KRAS mutation likely and may be helpful to direct molecular testing if targeted therapy is being considered. Also, TSAs are included in polyp counts to diagnose serrated polyposis syndrome, whereas TVAs are not. In our opinion sTVAs should not be included as part of this syndrome. Finally, it is only through precise histological diagnosis that the molecular spectrum of colorectal neoplasia can be fully elucidated. Thus strict diagnosis is an important part of ongoing research.

This paper does have limitations. Firstly, we set clear boundaries to separate the 3 polyp cohorts and this is reflected in the excellent level of diagnostic reproducibility. In practice a small subset of polyps will fall somewhere between the criteria we have used in this study and will remain difficult to precisely classify. However, in a proof of principle study such as this, we believe it is best to begin with clearly defined cohorts and strict diagnostic criteria. These can be refined over time as more information comes to light. Secondly, we do not have follow-up information on the patients included in this study to determine the clinical significance of these lesions in terms of the risk of subsequent polyps and colorectal carcinoma. The presence of frequent advanced histology would suggest close follow-up is warranted but needs to be addressed in a dedicated study in the future.

In summary, we find that sTVAs can be reliably diagnosed and represent an important precursor of KRAS mutated, MSS subtype colorectal carcinoma. In particular, KRAS mutation appears to be associated with the development of morphological serration. Serrated TVAs should not be diagnosed as TSAs due to significant differences in their underlying biology.
We gratefully acknowledge NHMRC grant funding (ID: 1063105). Mark Bettington gratefully acknowledges PhD scholarship funding from Cancer Council Queensland. MB, NW, CR, IB, AC, BL and VW collaborated to develop the study design. KK designed the statistical component of the study and performed the statistical analyses. MB, SP and DM performed the experiments. MB, NW, CR, IB and AC performed the histopathological analyses. MB, SP, DM and VW interpreted the data. MB wrote the manuscript. NW, CR, IB, BL and VW amended the manuscript.
REFERENCES


<table>
<thead>
<tr>
<th>Cohort</th>
<th>Non-advanced</th>
<th>Advanced</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low-grade dysplasia</td>
<td>High-grade dysplasia</td>
<td>Carcinoma</td>
<td>Total</td>
</tr>
<tr>
<td>Cohort 1</td>
<td>19 (70%)</td>
<td>8 (30%)</td>
<td>0</td>
<td>8 (30%)</td>
</tr>
<tr>
<td>(n=27)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cohort 2</td>
<td>0</td>
<td>19 (90%)</td>
<td>7 (33%)</td>
<td>21 (100%)*</td>
</tr>
<tr>
<td>(n=21)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cohort 3</td>
<td>46</td>
<td>4 (8%)</td>
<td>0</td>
<td>4 (8%)</td>
</tr>
<tr>
<td>(n=50)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cohort 4</td>
<td>50</td>
<td>13 (20%)</td>
<td>6 (9%)</td>
<td>16 (24%)*</td>
</tr>
<tr>
<td>(n=66)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Some cases in cohorts 2 and 4 had both high-grade dysplasia and carcinoma.
Table 2. Clinicopathological and molecular features of the study and control cohorts.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Serrated TVA (n=27)</th>
<th>Conventional TVA (n=50)</th>
<th>P-value</th>
<th>BRAF wild-type TSA (n=66)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>63.4</td>
<td>59.9</td>
<td>0.2460</td>
<td>63.8</td>
<td>0.9163</td>
</tr>
<tr>
<td>Female</td>
<td>9 (33%)</td>
<td>22 (44%)</td>
<td>0.4667</td>
<td>34 (52%)</td>
<td>0.1687</td>
</tr>
<tr>
<td>Size</td>
<td>21.6</td>
<td>13.4</td>
<td>&lt;0.0001</td>
<td>18.9</td>
<td>0.4684</td>
</tr>
<tr>
<td>Distal</td>
<td>16 (59%)</td>
<td>45 (90%)</td>
<td>0.0027</td>
<td>57 (89%)</td>
<td>0.0104</td>
</tr>
<tr>
<td>Advanced</td>
<td>8 (30%)</td>
<td>4 (8%)</td>
<td>0.0200</td>
<td>16 (24%)</td>
<td>0.6089</td>
</tr>
<tr>
<td>BRAF mutant</td>
<td>0</td>
<td>0</td>
<td>NA</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>KRAS mutant</td>
<td>18 (67%)</td>
<td>9 (18%)</td>
<td>&lt;0.0001</td>
<td>43 (65%)</td>
<td>1.0000</td>
</tr>
<tr>
<td>CIMP high</td>
<td>1 (4%)</td>
<td>0</td>
<td>0.3506</td>
<td>11 (17%)</td>
<td>0.1694</td>
</tr>
<tr>
<td>CIMP low</td>
<td>6 (22%)</td>
<td>3 (6%)</td>
<td>0.0590</td>
<td>30 (46%)</td>
<td>0.0594</td>
</tr>
<tr>
<td>CIMP negative</td>
<td>20 (74%)</td>
<td>47 (94%)</td>
<td>0.0279</td>
<td>25 (38%)</td>
<td>0.0026</td>
</tr>
<tr>
<td>MGMT methylation</td>
<td>12 (44%)</td>
<td>20 (40%)</td>
<td>0.8097</td>
<td>9 (14%)</td>
<td>0.0023</td>
</tr>
</tbody>
</table>

P-values of less than 0.05 are indicated in bold; TVA – tubulovillous adenoma; TSA – traditional serrated adenoma; CIMP – CpG island methylator phenotype
<table>
<thead>
<tr>
<th>Immunohistochemical stain</th>
<th>Serrated TVA (n=27)</th>
<th>Conventional TVA (n=50)</th>
<th>P-value</th>
<th>BRAF wild-type TSA (n=66)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear β-catenin</td>
<td>19 (70%)</td>
<td>42 (84%)</td>
<td>0.2384</td>
<td>16 (24%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>P53</td>
<td>4 (15%)</td>
<td>3 (6%)</td>
<td>0.2322</td>
<td>12 (18%)</td>
<td>0.7723</td>
</tr>
<tr>
<td>P16</td>
<td>24 (89%)</td>
<td>40 (80%)</td>
<td>0.5248</td>
<td>52 (79%)</td>
<td>0.3772</td>
</tr>
<tr>
<td>MLH1 loss</td>
<td>0</td>
<td>0</td>
<td>1.000</td>
<td>0</td>
<td>1.000</td>
</tr>
<tr>
<td>Ki67</td>
<td>9 (33%)</td>
<td>45 (90%)</td>
<td>&lt;0.0001</td>
<td>47 (71%)</td>
<td>0.0010</td>
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<tr>
<td>CK7</td>
<td>1 (4%)</td>
<td>8 (16%)</td>
<td>0.1488</td>
<td>27 (41%)</td>
<td>0.0003</td>
</tr>
<tr>
<td>CK20</td>
<td>23 (85%)</td>
<td>46 (92%)</td>
<td>0.4404</td>
<td>65 (99%)</td>
<td>0.0238</td>
</tr>
</tbody>
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P-values of less than 0.05 are indicated in bold.
Table 4. Immunohistochemical features of low-grade dysplasia components versus advanced components of the 48 serrated tubulovillous adenomas

<table>
<thead>
<tr>
<th>Immunohistochemical stain</th>
<th>Low-grade dysplasia (n=19)</th>
<th>High-grade dysplasia/colorectal carcinoma (n=29)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear β-catenin</td>
<td>10 (53%)</td>
<td>23 (79%)</td>
<td>0.064</td>
</tr>
<tr>
<td>P53</td>
<td>2 (11%)</td>
<td>12 (41%)</td>
<td><strong>0.0264</strong></td>
</tr>
<tr>
<td>P16</td>
<td>16 (84%)</td>
<td>28 (97%)</td>
<td>0.2864</td>
</tr>
<tr>
<td>MLH1 loss</td>
<td>0</td>
<td>0</td>
<td>1.000</td>
</tr>
<tr>
<td>Ki67</td>
<td>9 (47%)</td>
<td>19 (66%)</td>
<td>0.2446</td>
</tr>
<tr>
<td>CK7</td>
<td>1 (5%)</td>
<td>2 (8%)</td>
<td>1.0000</td>
</tr>
<tr>
<td>CK20</td>
<td>15 (79%)</td>
<td>21 (72%)</td>
<td>0.7395</td>
</tr>
</tbody>
</table>

P-values of less than 0.05 are indicated in bold.
FIGURE LEGENDS

Figure 1. (A) A low power image of a serrated tubulovillous adenoma. (B) A medium power image of the polyp in (A) showing prominent undulation of the glandular epithelium. (C) Ectopic crypt formations (arrows) with absence of traditional serrated adenoma cytology. This is the same polyp as illustrated in figure 3. (D) A medium power image from the polyp in (A) showing ‘maze-like’ growth with prominent arborizing and right-angled branching of the glandular epithelial lumens.

Figure 2. (A and C) Low and medium magnification images of a conventional tubulovillous adenoma. Note the lack of epithelial serration. (B and D) Low and medium magnification images of a traditional serrated adenoma. Note the characteristic cytology, abundant ‘slit-like’ epithelial serrations (arrows) and ectopic crypt formations.

Figure 3. (A) Low magnification image of a serrated tubulovillous adenoma giving rise to an invasive adenocarcinoma. (B) High magnification image of the carcinoma showing serrated morphology, i.e. serration of the epithelium, abundant eosinophilic cytoplasm, basally located vesicular nuclei, prominent nucleoli and lack of dirty necrosis.

Figure 4. β-catenin immunohistochemistry. (A) Serrated tubulovillous adenoma showing moderate to strong nuclear staining in most of the epithelial cells. (B) A non-advanced traditional serrated adenoma showing membranous staining but lack of nuclear staining (the normal pattern).

Figure 5. Proposed precursors and pathways of sporadic colorectal carcinogenesis.
Chapter 6 – Final Discussion:
The serrated neoplasia pathway is a relatively recently described molecular route to colorectal carcinoma that accounts for 20-30% of all colorectal carcinoma.\textsuperscript{1,2} As such the serrated pathway is a major source of gastrointestinal cancer and a major health and economic burden. While the precursors of the conventional pathway to colorectal carcinoma are well characterised, the precursors of the serrated pathway are much less understood. This has significant implications, as endoscopic removal of the pre-malignant polyps is an effective method to prevent the development of cancer. Thus a detailed understanding of serrated colorectal polyps may allow for more informed decisions about the management of serrated polyps.

Chapter 2 addresses two important issues. First we assessed a large consecutive series of colorectal polyps with the primary aim of determining how frequent sessile serrated adenomas are in our community. Second, through a detailed histological and clinical appraisal we attempted to provide evidence for the diagnostic threshold of the sessile serrated adenoma. These issues are related and are important for several reasons. For pathologists it is important to know how frequently we should be rendering specific diagnoses. At present the rates of sessile serrated adenoma diagnoses in the literature are widely variable,\textsuperscript{3-9} making it difficult for the practicing pathologist to know what is reasonable. Furthermore, the diagnostic threshold for the sessile serrated adenoma continues to change, with a definite trend to relaxed criteria.\textsuperscript{10,11} However these decisions have been made mostly on the basis of expert opinion rather than based on experimental evidence.

In our practice, we were using the diagnostic criteria of the 4th edition of the WHO classification of tumours of the digestive system.\textsuperscript{10} We felt that we were diagnosing sessile serrated adenomas more frequently than had been reported in the literature. In addition, a consensus paper from a panel of international experts had recently suggested relaxing the diagnostic criteria beyond the recommendations of the WHO.\textsuperscript{11} Thus we undertook to determine our rate of sessile serrated adenoma diagnoses and to compare the clinical features of sessile serrated adenomas when divided according to the number of sessile serrated adenoma-type crypts per polyp. We found that polyps with even one typical sessile serrated adenoma-type crypt had gender and distribution characteristics more similar to other more ‘typical’ sessile serrated adenomas than to microvesicular hyperplastic polyps. Using this diagnostic threshold, we found that sessile serrated
adenomas represented 14.7% of all colorectal polyps received at our practice. This figure represents the highest in the reported literature. This result likely reflects the relaxed diagnostic threshold utilised but probably also reflects the experience of the referring colonoscopists. Brisbane has been a centre of research into the serrated neoplasia pathway for many years and as such local gastroenterologists may be more aware of the salient features of sessile serrated adenomas than in other regions. As such the high rate of sessile serrated adenomas reported in our series may not be entirely reflective of the experience of other centres. Regardless, sessile serrated adenomas should be a common diagnosis to both gastrointestinal and general anatomical pathologists.

Interval colorectal carcinoma accounts for approximately 5% of all colorectal carcinoma diagnoses and is defined as a cancer developing within the colonoscopic surveillance interval. Serrated pathway carcinomas are over-represented in series of interval colorectal carcinomas. There are several reasons why this may be the case. First are missed polyps at colonoscopy. Sessile serrated adenomas are predominantly proximal and can be very subtle, particularly when compared to conventional adenomas. Second, there is a hypothesised potential for sessile serrated adenomas to progress rapidly to malignancy. Third, sessile serrated adenomas are frequently incompletely excised at colonoscopy. It is probable that all of these factors play some role in the occurrence of interval colorectal carcinoma. Another factor that had not been previously reported is under-diagnosis of sessile serrated adenomas by pathologists. The diagnosis of the sessile serrated adenoma overlaps with microvesicular hyperplastic polyps. Microvesicular hyperplastic polyps are benign and do not have significant malignant potential. As such no specific surveillance is recommended for these polyps. Overly stringent diagnostic criteria will result in a proportion of sessile serrated adenomas being diagnosed as microvesicular hyperplastic polyps with the potential for inadequate recommendations for surveillance colonoscopy and thus contributing to the development of interval colorectal carcinoma.

We hope that the finding of chapter 2 will assist pathologists by reinforcing that sessile serrated adenomas are common and by providing some basic evidence for the relaxed diagnostic criteria proffered by the expert panel.

Chapter 3 is a detailed analysis of the traditional serrated adenoma. These rare polyps have not been thoroughly categorised. In this chapter we detail the clinicopathological and molecular features of the traditional serrated adenoma and provide insights into the
molecular pathways by which traditional serrated adenomas progress to colorectal carcinoma. At present, there are several issues of contention regarding these lesions to the extent that some authors do not accept that the traditional serrated adenoma is part of the serrated neoplasia pathway. In some countries, traditional serrated adenomas are considered to be exclusively distal and are required to have a protuberant or exophytic growth pattern for diagnosis. Many series from various regions have described precursor microvesicular hyperplastic polyp or sessile serrated adenomas at the edge or base of traditional serrated adenomas, however this is still not broadly accepted. Furthermore, the rates of KRAS and BRAF mutation and CpG island methylator phenotype of traditional serrated adenomas is widely variable in the literature. Finally the molecular steps in malignant transformation have not been thoroughly assessed.

In our series we aimed to address these issues. Our series comprised 200 traditional serrated adenomas with strict inclusion criteria. We identified slit-like serrations as a very helpful histological feature for making the diagnosis of traditional serrated adenoma, and in particular for separating these polyps from serrated tubulovillous adenomas. In addition we were able to perform a clinicopathological and molecular assessment of all of the cases included in the study. The rigorous inclusion criteria, the size of the series and the complete assessment of all of the cases allowed us to identify statistically significant features of traditional serrated adenomas that had not been previously recognised.

We found that most traditional serrated adenomas harbour a BRAF mutation and that the dichotomy between BRAF mutated and KRAS mutated traditional serrated adenomas underlies important differences in the clinicopathological and molecular features of these polyps. In particular, BRAF mutated traditional serrated adenomas frequently arise from either a microvesicular hyperplastic polyp or sessile serrated adenoma, whereas KRAS mutated cases do not. Second, BRAF mutated traditional serrated adenomas regularly occur in the proximal colon. These proximal traditional serrated adenomas are often sessile and this may relate simply to a difference in intraluminal pressures rather than to any intrinsic factor of the polyps, especially because distal BRAF mutated traditional serrated adenomas are no more likely to be flat than the distal KRAS mutated traditional serrated adenomas. Third, the BRAF mutated traditional serrated adenomas are much more likely to be CpG island methylator phenotype high. This is expected as BRAF mutation is known to be tightly correlated with the CpG island methylator phenotype. More recently a mechanistic link between BRAF mutation and methylation has been
demonstrated. Finally, \textit{BRAF} mutated traditional serrated adenomas showed much more frequent loss of expression of p16 by immunohistochemistry than other cases and likely reflects the methylator phenotype of the \textit{BRAF} mutated polyps.

Thirty-eight of the cases in this series had developed an area of either overt cytological dysplasia or of malignancy. Interestingly, loss of mismatch repair enzyme function was very rare, even amongst the \textit{BRAF} mutated cases. This has been described by other groups and represents an important distinction in the mode of progression of traditional serrated adenomas compared to sessile serrated adenomas. As discussed above, \textit{BRAF} mutated polyps were more likely to lose immunohistochemical expression of p16, but the rates in \textit{BRAF} and \textit{KRAS} mutated lesions for \textit{TP53} mutation and WNT pathway activation were very similar.

\textit{BRAF} mutated microsatellite stable colorectal carcinomas are known to have a poor prognosis. Fortunately these cancers are rare, accounting for approximately 5% of all colorectal cancer. \textit{BRAF} mutated traditional serrated adenomas appear to be an important precursor of these cancers. Thus, although they are rare, traditional serrated adenomas give rise to an aggressive subtype of colorectal carcinoma. Thus any patient with a traditional serrated adenoma with overt cytological dysplasia should be closely followed by colonoscopy. Whenever carcinoma is found to be arising in a traditional serrated adenoma, partial colectomy should be seriously considered regardless of the presence or absence of other adverse factors.

Chapter 4 addressed the same aims as chapter 3, but this time focussing on advanced sessile serrated adenomas. Unlike traditional serrated adenomas, sessile serrated adenomas are common and as such account for the bulk of the cancer burden of the serrated neoplasia pathway. As has already been discussed, they are also likely to be the cause of many interval carcinomas. Despite the frequency of sessile serrated adenomas, cases with dysplasia or early carcinoma, i.e. sessile serrated adenomas “caught in the act” of malignant transformation, are rare. As a result there have been no thorough studies of a large series of polyps of this type.

Although much can be inferred about the clinicopathological and molecular features of these polyps by study of either ordinary sessile serrated adenomas or of \textit{BRAF} mutated colorectal carcinomas, inevitable gaps will remain. Particular issues that cannot be
resolved include the proportion of cases that are mismatch repair deficient versus mismatch repair proficient, the size of the polyps when they develop dysplasia or carcinoma and the nature of the dysplasia (size, growth pattern). In addition, study of \textit{BRAF} mutant colorectal carcinomas will not capture the small but significant subset of \textit{BRAF} wild type sessile serrated adenomas.

For this chapter we collected a prospective series of 137 advanced sessile serrated adenomas. Importantly all of the cases were rigorously diagnosed, paying particular attention to exclusion of tubular adenoma contaminating an ordinary sessile serrated adenoma and the more recently recognised traditional serrated adenoma (or early traditional serrated adenoma-type change) arising in a sessile serrated adenoma. Other studies that are based on database review, without central review and without strict histopathological criteria must be interpreted with caution.

As a group, the advanced sessile serrated adenomas tended to be small polyps. The median size was 9.5mm. Most occurred in the proximal colon and in women. In the majority the advanced component was flat. We found that 75% of advanced sessile serrated adenomas are mismatch repair deficient. This occurs via silencing of the \textit{MLH1} gene by methylation of the promoter region.\textsuperscript{33} Thus loss of \textit{MLH1} function was the most common molecular event in the neoplastic progression of these polyps. Furthermore, the mismatch repair enzyme status underscored key differences in the clinicopathological and other molecular features of these polyps.

Mismatch repair deficient polyps occurred more frequently in the proximal colon and more often in women. They were more likely to be CpG island methylator phenotype high and were less likely to have a \textit{TP53} mutation. Loss of p16 expression and WNT pathway activation were similar between the two groups.

These findings are important. The proximal location, small size of these polyps and the frequent flat nature of the advanced components have implications for colonoscopists. Proximal polyps are more difficult to detect and remove than distal ones due to technical issues and the anatomy of the colon.\textsuperscript{34} Adding to the difficulty, sessile serrated adenomas are subtle. They are close in colour to the surrounding normal mucosa, often have indistinct borders and the type II pit pattern is not as striking as the type III and IV pattern of conventional adenomas.\textsuperscript{35, 36} In addition they are often obscured by mucin or adherent
debris. While this can be a helpful feature to the knowledgeable, lack of awareness of this phenomenon is problematic for others. Finally, the frequent small size of these polyps means they can be easily missed. Thus even diligent and knowledgeable colonoscopists will inevitably miss a proportion of sessile serrated adenomas. If the missed lesion contains a focus of dysplasia or early carcinomas this can easily become an interval carcinoma. Sessile serrated adenomas with dysplasia have a hypothesised potential for rapid malignant transformation. Although not proven, indirect evidence supports this position. First, the majority have lost mismatch repair enzyme function allowing for a rapid accumulation of mutations in tumour suppressor genes with mono or bi-nucleotide repeats.\(^{37,38}\) Second, the CpG island methylator phenotype allows progressive silencing of important tumour suppressor genes. Third sessile serrated adenomas are common and BRAF mutated colorectal carcinomas are common, but advanced sessile serrated adenomas are rare.\(^{39}\) This would suggest that they are present only briefly in the large bowel before progressing to cancer. Finally, the mean age of patients in this series with sessile serrated adenomas with dysplasia was not significantly different to cases with cancer, but both were significantly older than the reported mean ages of ordinary sessile serrated adenomas in the literature.\(^{39,40}\) This suggests that the lag between dysplasia and cancer is very short. Thus whenever an advanced sessile serrated adenoma is missed, there is a substantial risk of interval cancer.

The separation of advanced sessile serrated adenomas into distinct mismatch repair deficient and mismatch repair proficient subsets has not been previously demonstrated; however the finding is not unexpected based known differences between BRAF mutated microsatellite unstable and stable carcinomas.\(^{31,32}\) As discussed above, BRAF mutated traditional serrated adenomas are an important precursor of the aggressive BRAF mutated microsatellite stable subtype of cancer, however the majority are probably derived from these mismatch repair proficient advanced sessile serrated adenomas. Thus up to one quarter of advanced sessile serrated adenomas will become the aggressive BRAF mutated, microsatellite stable molecular subtype of cancer and these will be over-represented amongst males and amongst distal polyps.

Chapter 5 evolved in part from the work on chapter 3. While gathering the series of traditional serrated adenomas we identified many polyps with tubulovillous growth and ectopic crypt formations. However, they did not have the typical cells with eosinophilic cytoplasm and did not show slit-like serrations. Instead they had uniform cytological
dysplasia of conventional type and other features of serration, such as undulation of the epithelium and a distinctive maze-like growth pattern. Some of these polyps had been diagnosed as traditional serrated adenomas based on the work of Torlakovic et al, from 2008.\textsuperscript{16} However, after central review they were excluded. It was unclear to us if these serrated tubulovillous adenomas should be classified as traditional serrated adenomas, tubulovillous adenomas or as a separate category. Thus we undertook to gather a consecutive series of these polyps, with appropriate control cohorts to assess their clinicopathological and molecular features.

We found that the serrated tubulovillous adenomas had distinctive histological features and could be reliably distinguished from both traditional serrated adenomas and from conventional tubulovillous adenomas. Furthermore the serrated tubulovillous adenomas had clinicopathological and molecular features distinct from the two control cohorts. In particular they had a very high rate of KRAS mutation suggesting that serrated morphology may be linked to KRAS mutation. This is a concept first raised by Jeremy Jass\textsuperscript{41} but that has not been conclusively demonstrated in an experimental setting. The serrated tubulovillous adenomas had relatively little methylation. It seems likely that these polyps are a progressed form of tubulovillous adenoma and that the prominent serrated architecture develops after the acquisition of a KRAS mutation; however this theory requires more intensive and directed investigation.

In conclusion this thesis has addressed many aspects of serrated colorectal polyps. We have determined the frequency of sessile serrated adenomas in a community pathology practice based on varied criteria for these polyps. In addition we have validated by clinical data the single crypt criteria for the diagnosis of sessile serrated adenomas. Next we have performed an in-depth clinicopathological and molecular appraisal of traditional serrated adenomas and advanced sessile serrated adenomas and have identified novel histological, clinicopathological and molecular features of these polyps, many of which are relevant to clinical practice. Finally we have assessed the features of a serrated variant of tubulovillous adenomas and have shown they have distinctive features warranting separation from traditional serrated adenomas.
24. Tsai JH, Liau JY, Lin YL, et al. Traditional serrated adenoma has two pathways of neoplastic progression that are distinct from the sessile serrated pathway of colorectal carcinogenesis. Mod Pathol. 2014.


