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A MORPHOLOGICAL AND MOLECULAR STUDY OF PROPOSED EARLY FORMS OF THE TRADITIONAL SERRATED ADENOMA

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ABSTRACT

Aims: The traditional serrated adenoma (TSA) is the least common subtype of serrated colorectal polyp. Large protuberant lesions are easily recognized, however, the origins of TSAs are not known and early forms have not been described. Some large TSAs present with a flat "shoulder" component surrounding the central protuberant component. We hypothesized that small polyps with the same histology as these "shoulder" regions may represent early TSAs.

Methods and results: We collected 70 small (< 10mm) polyps that may represent early TSAs based on typical TSA cytology covering the luminal surface. We also identified 12 large TSAs with a "shoulder" component resembling these small polyps. The study polyps patients had a mean age of 58 years, 54% were female, had a mean 4.1mm diameter and were predominantly distal (71%). Morphologically, slit-like serration was present in 81%, ectopic crypt formations in 67% and a villous component in 47%. These histological features were similar to the 12 "shoulder" lesions. Immunohistochemical stains showed absence of β-catenin nuclear expression in 96% of the small polyps, retained expression of MLH1 in 100% and Ki-67 positivity restricted to the crypt bases and ectopic crypt formations. *BRAF* and KRAS mutations were identified in 47% and 31% of the polyps respectively. Compared with *KRAS*-mutated polyps, *BRAF*-mutated polyps were more likely to arise in a precursor polyp (82% vs 18%, $P < 0.001$) and were more likely to have slit-like serrations (100% vs 73%, $P = 0.003$).

Conclusions: These morphological, immunohistochemical and molecular findings are similar to what has been reported in large TSAs and support the hypothesis that these polyps represent early forms of TSA.

Key words: Colonic polyps; Traditional serrated adenoma; Serrated polyp; Immunohistochemistry; BRAF; KRAS

INTRODUCTION

Serrated colorectal polyps fall into three major categories, namely hyperplastic polyps (HPs), sessile serrated polyps (SSPs) and traditional serrated adenomas (TSAs) [1]. Of these only SSPs and TSAs have significant malignant potential. Since the first report of the TSA by Longacre and Fenoglio-Presier [2], our understanding of the histology and molecular biology of this rare polyp continues to improve. Clinically, TSAs tend to occur in older patients, more commonly in the distal colon and rectum and have no significant gender predilection [3-5]. Histologically, the classic TSA is characterised by tubulovillous architecture, ectopic crypt formations, slit-like serrations and typical cytology (cells with abundant intensely eosinophilic cytoplasm and centrally placed pencillate nuclei). Although not universally recognised, proximal TSAs that are often flat to the endoscopist, have been described [3, 4]. At a molecular level most TSAs demonstrate activation of the MAP kinase pathway by either *KRAS* mutation in 22-42% or *BRAF* mutation in 48-67% [4-7]. A small proportion are *BRAF* / *KRAS* wild-type, and these cases segregate best with the *KRAS* mutated group [3]. WNT pathway activation by *PTPRK-RSPO3* fusions or *RNF43* mutations occur in 71% of TSAs [8]. Compared with *KRAS*-mutated TSAs, *BRAF*-mutated TSAs are more likely to be proximal, to arise in a precursor polyp and to show the CpG island methylator phenotype [3, 9]. Regardless of the *BRAF* or *KRAS* mutation status, effectively all TSAs are mismatch repair proficient, even when they develop high grade dysplasia or malignancy [3].

Despite these recent studies, there are still several controversial aspects to TSAs. Proximal and flat TSAs remain a matter of contention. The origin of TSAs is also not clear. While many *BRAF*-mutated TSAs appear to have their origins in microvesicular hyperplastic polyps or SSPs, this is not universally accepted [3, 4, 7, 10]. The origins of *KRAS*-mutated TSAs remain even more obscure. In our practice we often see large distal TSAs with a central protuberant portion but with a collar (or shoulder) of flat TSA at the periphery. Furthermore, we sometimes see small polyps with features very similar to these "shoulder" areas of larger TSAs. Because there is at present no good alternative, pathologists tend to diagnose these as tubular adenomas (albeit odd-looking) or sessile serrated polyps with dysplasia (SSPD) when an adjacent SSP component is present. In the current literature, most described TSAs are greater than 10mm in size. However, TSAs cannot arise as large polyps. Despite this, there are currently no descriptions dedicated to small TSAs.

We hypothesised that early forms of the TSA are recognisable and will have morphological, immunohistochemical and molecular features similar to the "shoulder" areas of large TSAs. Thus the aims of the current study were to 1) describe the histology of a series of small polyps that may represent early forms of TSAs, 2) to perform a basic immunohistochemical (Ki-67, β-catenin, MLH1) and molecular (*BRAF* and *KRAS* mutation status) panel on these polyps and to compare to a control group of "shoulder areas" of larger TSAs, and 3) to identify clinicopathological differences between *BRAF*- and *KRAS*-mutated early TSAs.

MATERIALS AND METHODS

Patients and Samples

The study group comprised cases collected in a prospective fashion by one of the authors (NW) during routine reporting at Envoi Specialist Pathologists in Brisbane, Australia, between January 2014

and June 2015. All cases were reviewed by two other pathologists (MB and CR). Demographic data including age, gender and polyp location were collected from the specimen request form or colonoscopy report. Distal location was assigned for polyps distal to the splenic flexure. A control group comprising the "shoulder" areas of larger TSAs was used for comparison to the study polyps and were selected from cases reported in a previous study [3]. Ethics approval for the study was given by the QIMR Berghofer Medical Research Institute (P1298).

Histological inclusion criteria

The study polyps were identified based on size (≤ 10mm) and histological features. The major histological feature was a polyp with TSA-type cytology (eosinophilic cells with centrally placed pencillate nuclei) covering the luminal surface. Any component of villous growth, ectopic crypt formations and slit-like serrations were noted, but not required for inclusion. Villous growth was defined as "a free-floating structure with epithelium surrounding a central core of lamina propria", an ectopic crypt formation was defined as "epithelial buds with their bases not in contact with the muscularis mucosa" and a slit-like serration as "a narrow slit in the epithelium, similar to those seen in normal small bowel" (Figure 1). In cases where there was a clear precursor polyp (e.g. microvesicular HP or SSP), the TSA-type cytology was only required to cover the luminal aspect of the discrete TSA-like area of these polyps.

Immunohistochemistry

Immunohistochemical stains for MLH1, β-catenin and Ki-67 were performed on the study and control polyps as previously described [3].

KRAS and *BRAF* mutation testing

Testing for *KRAS* and *BRAF* mutations was performed on the study and control polyps as previously described [3].

Statistical analysis

Categorical variables were compared using Fisher's exact test. Continuous variables were compared using Student's *t*-test. A *P*-value of < 0.05 was considered significant.

RESULTS

Clinicopathological features of early traditional serrated adenomas

Twelve control polyps with an adequate "shoulder" region for comparison were identified from the previously published cases [3] (figure 2). A further 70 polyps met the inclusion criteria for the study (figure 3). The clinicopathological features of the study polyps are presented in table 1. The mean age of the patients was 58 years and the mean polyp size 4.1 mm. Thirty-eight (54%) of the patients were female.

The histological and immunohistochemical features are described in table 2. For inclusion, all cases required TSA-type cytology covering the luminal aspect of the polyp. A precursor polyp was identified in 34 cases (49%), comprising 13 (19%) microvesicular HPs and 16 (22%) SSPs. Thirty-three (47%) had at least focal villous architecture, 57 (81%) slit-like serrations and 47 (67%) ectopic crypt formations. By comparison, the "shoulder" areas of the control cohort showed focal villous change in 3 (25%), slit-like serrations in 9 (75%) and ectopic crypt formations in 7 (58%). There was no statistically significant difference between the study polyps and the control group (apart from origin in a precursor polyp).

Immunohistochemical and molecular features

The molecular features are described in table 3. All of the study and control polyps retained nuclear staining for MLH1 (mismatch repair proficient) (figure 4D). Nuclear staining for β-catenin was either absent in 67 cases (96%) (figure 4B) or very focal in 3 cases (4%). The three cases with focal positive staining were limited to occasional ectopic crypts, a staining pattern previously described [11]. Ki-67 showed the same pattern of staining in all study and control cases, with prominent staining in the crypts and in ectopic crypt formations but absent staining at the luminal surface (figure 4C). There were no statistically significant differences in the immunohistochemical profiles of the study or control polyps.

Of the 70 study polyps, 33 (47%) were *BRAF*-mutated, 22 (31%) *KRAS*-mutated and the remainding 15 cases were wild-type for both genes. All 12 of the control polyps were *KRAS*-mutated. The clinicopathological features of the *KRAS*- versus *BRAF*-mutated polyps are presented in table 3. *BRAF*-mutated polyps were more likely to arise in a precursor polyp (82% vs 18%, $P < 0.001$) and were more likely to have slit-like serrations than *KRAS*-mutated polyps (100% vs 73%, $P = 0.003$) (Table 3).

DISCUSSION

The TSA has been described as the least common and least understood subtype of the serrated polyps [12]. Currently it is unusual to diagnose TSA in a polyp under 10mm, but logically early and small forms of these polyps must exist. The aim of this study was to attempt to identify early forms of the TSA. In all, 70 lesions measuring less than 10mm (mean 4.1 mm) were selected. We reasoned that if these early putative TSAs shared morphological and immunohistochemical features with the "shoulders" of the larger TSAs, then this would add support to the hypothesis that these are in fact small TSAs. Morphologically, there were no statistically significant differences between the "shoulder" areas of large TSAs and the small TSAs of the study group. Furthermore, the staining

patterns for MLH1, Ki-67 and β-catenin were the same between both groups. In particular all cases were mismatch repair proficient, lacked Ki-67 staining at the luminal surface and nearly uniformly lacked nuclear staining for β-catenin.

This immunohistochemical pattern is in stark contrast to those described for both SSPDs and conventional tubular adenomas, the two main differential diagnoses of early TSA. *BRAF*-mutated early TSAs arising in a precursor microvesicular HP or SSP can be confused with SSPD. This distinction may be clinically relevant. The SSPD is an advanced polyp at a molecular level, with the potential for rapid transition to malignancy, in contrast an early TSA is a relatively benign polyp with less immediate malignant potential [13]. The dysplastic portion of an SSPD should show overt cytological dysplasia with frequent mitoses, including at the luminal surface [14]. This is not a feature of TSA arising in a microvesicular HP or SSP. In addition, SSPD typically show strong staining for Ki-67 at the luminal surface in the areas of dysplasia, concordant with the atypical histology [15]. Nuclear β-catenin is also relatively common in the dysplastic areas, reflecting abnormalities in the WNT signalling pathway and up to 75% lose staining for MLH1 [13-15]. *KRAS*-mutated TSAs rarely arise in a precursor polyp and can resemble conventional tubular adenomas [10]. However, tubular adenomas are overtly dysplastic, show increased Ki-67 staining at the luminal surface and frequently show nuclear β-catenin staining [16, 17]. Kim et *al*. previously reported "tubular adenoma-like" lesions in 18% of TSAs as endoscopically flat-elevated lesions demonstrating differences in morphology and distribution of Ki-67 to conventional tubular adenomas [4]. Furthermore, MAP kinase pathway activation is rare in small tubular adenomas [17].

These findings, of striking histological and immunohistochemical similarity between the study polyps and the shoulder areas of the larger TSAs and the marked differences to the histology and immunohistochemical staining profiles of SSPD and tubular adenomas support the hypothesis that these small polyps may represent early forms of the TSA. However, it must be acknowledged, that in

diminutive cases with no precursor polyp, separation from a conventional tubular adenoma in routine reporting would be impractical and of limited clinical utility. Application of immunohistochemistry may resolve the uncertainty in most of these cases, but given the limited clinical significance is not justified. However, once slit-like serrations and ectopic crypt formations become identifiable, the diagnosis of a TSA should be considered, even if villous change is not apparent.

Of the 70 polyps included in this study, 33 were *BRAF*-mutated, 22 *KRAS*-mutated and the remainder *BRAF* / *KRAS* wild-type. These ratios are consistent with the published literature [3-5, 9, 11]. The major difference between the groups was the very frequent origin of the *BRAF*-mutated cases in a microvesicular HP or SSP. Previously microvesicular HP or SSP has been associated with 15-57% of *BRAF*-mutated TSAs [3-5]. As might be expected, smaller lesions would be less likely to have overgrown their precursor and therefore it is predictable that the association would be higher. While it is likely the remaining polyps overgrew a pre-existing lesion, a rare de novo polyp cannot be excluded. In contrast, a precursor was uncommon amongst *KRAS*-mutated cases. However, four did show an adjacent goblet cell HP, suggesting that at least some may arise in these polyps. If *KRAS*-mutated hyperplastic, non-serrated aberrant crypt foci give rise to goblet cell hyperplastic polyps as suggested [18], then perhaps they can also directly give rise to *KRAS*-mutated TSAs.

Recently a Japanese group has published a series of polyps designated as "superficially serrated adenoma"[19]. These polyps appear very similar morphologically to the *KRAS*-mutated early TSAs in our series. Interestingly, 19 of their 20 cases harboured a *KRAS* mutation and 18 of the 20 cases showed either *RSPO3* fusion or over-expression, a feature identified in up to one third of larger TSAs. In the same study, the authors retrospectively identified large TSAs with "superficially serrated adenoma" at the edges (very similar in appearances to the "shoulder" areas of our large TSAs) and found *RSPO3* fusion or over-expression in 14 of 15 cases. Although the final interpretation by the authors was that

these represent a separate precursor lesion to the TSA, in our opinion the morphology and molecular alterations are more suggestive of an early form of TSA. Regardless, there is a clear morphological and molecular relationship between these lesions and the TSA, quite distinct from the alternative diagnoses of goblet cell HP, SSPD or tubular adenoma.

In summary, we reported a series of 70 small polyps that we feel represent the early forms of the large well-recognized TSA. At present, many of these polyps would be diagnosed as "odd-looking" tubular adenoma (*KRAS*-mutated forms) or possibly as SSPDs (*BRAF*-mutated forms). Careful attention to the morphology, in particular, TSA-type cytological changes with concurrent slit-like serrations and ectopic crypt formations, can be recognised as clues to the diagnosis. Prospective studies are needed to understand the clinical significance of these lesions. It is currently unknown if a three-year colonoscopy surveillance interval, as currently recommended for the usual TSA, is justified for early forms of TSA.

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Table 1. Clinicopathological features of the study and control polyps

	Study polyps (n=70)	Control polyps (n=12)	P-value
Age, years (mean, range)	58 (23-85)	69 (43-84)	0.02
Female	38 (54%)	9 (75%)	0.22
Distal location	50 (71%)	12 (100%)	N/A
Size, mm (mean, range)	4.1 (1-10)	23.7 (8-50)	N/A

Table 2. Histological and immunohistochemical features of the study polyps

	All (n=70)	*BRAF* mutated (n=33)	*KRAS* mutated (n=22)	Control group (n=12)	p-value (*BRAF* versus *KRAS* mutated)
Villous component	33 (47%)	18 (55%)	10 (45%)	3 (25%)	0.59
Ectopic crypt formations	47 (67%)	29 (88%)	16 (73%)	7 (58%)	0.17
Slit-like serrations	57 (81%)	33 (100%)	16 (73%)	9 (75%)	0.003
Precursor polyp	34 (49%)	27 (82%)	4 (18%)	0	0.0001
- MVHP	12 (17%)	12 (36%)	0	0	0.0016
- SSA	16 (23%)	15 (45%)	0	0	0.0001
- GCHP	6 (9%)	0	4 (18%)	0	0.0214
Nuclear β-catenin staining	3 (4%)	0%	3 (14%)	2 (17%)	0.06
Retained MLH1 staining	70 (100%)	33 (100%)	22 (100%)	12 (100%)	1.000
Ki67	Descriptive report only				

MVHP – microvesicular hyperplastic polyp; SSA – sessile serrated adenoma; GCHP – goblet cell hyperplastic polyp

Table 3. Clinicopathological features of the study by mutation status

	BRAF mutated polyps (n=33)	*KRAS* mutated polyps (n=22)	P-value
Age, years (mean, range)	56 (36-89)	69 (43-84)	0.02
Female	18 (54%)	75%	0.22
Distal location	23 (71%)	100%	N/A
Size, mm (mean, range)	4.8 (2-10)	4.1 (2-8)	NA

Figure Legends

Figure 1. (A) Slit-like serration is defined as narrow slit in the epithelium, similar to those seen in normal small bowel. (B) Ectopic crypt formations are epithelial buds with their bases not in contact with the muscularis mucosa.

Figure 2. (A and B) Low power examples of the shoulder areas from the large *KRAS*-mutated control traditional serrated adenomas. (C and D) Medium and high power of the shoulder areas showing typical cytology and slit-like serrations, (C) has some rudimentary ectopic crypt formations.

Figure 3. (A) An early *BRAF*-mutated traditional serrated adenoma arising in a microvesicular hyperplastic polyp. The central traditional serrated adenoma component has typical cells with eosinophilic cytoplasm and bland pencillate nuclei. Some villous change is present and numerous slit-like serrations are seen. Occasional ectopic crypt formations are also evident. (B) Another example of an early *BRAF*-mutated traditional serrated adenoma, but this example has a flat surface profile. (C and D) Examples of early *KRAS*-mutated traditional serrated adenomas with typical cytology and slit-like serrations, (D) has some rudimentary ectopic crypt formations. (E) A particularly villiform early *BRAF*-mutated traditional serrated adenoma.

Figure 4. (A-D) Haematoxylin and eosin, β-catenin, Ki67 and MLH1 from a *KRAS*-mutated early traditional serrated adenoma. Note the absence of nuclear staining for β-catenin, the lack of surface Ki67 proliferative activity and the uniform retained nuclear staining for MLH1.