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Agrin in the Muscularis Mucosa Serves as a Biomarker Distinguishing Hyperplastic Polyps from Sessile Serrated Lesions

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Abstract

Purpose: Sessile serrated lesions (SSL) are precursors to colon carcinoma (CRC), and their distinction from other polyps, in particular hyperplastic polyps (HPs), presents significant diagnostic challenges. We evaluated expression patterns in colonic polyps of previously identified CRC-associated extracellular matrix (ECM) proteins to identify markers distinguishing SSLs from other polyps.

Experimental Design: Gene-expression analyses of ECM proteins were performed using publicly available data on pre-neoplastic colonic polyps. In parallel, we evaluated by immunohistochemistry the expression of agrin (AGRN) in over 400 colonic polyps, including HP, SSL with and without dysplasia, traditional serrated adenomas (TSA), tubular adenomas (TA) and compared the consistency of standard histological diagnosis of SSLs by experienced gastrointestinal pathologists with that of AGRN immunohistochemistry.

Results: Differential gene expression analysis and immunohistochemistry identified AGRN, serine peptidase inhibitor (SERPINE2) and TIMP metalloproteinase inhibitor 1 (TIMP1) elevated in SSLs and HPs but decreased in TAs and absent in normal colon. AGRN-positive basal laminae (BL) were noted in all TA, TSA, HP and SSL in distinguishable patterns, whereas other polyps and normal mucosa were negative. SSL with or without dysplasia consistently showed immunohistochemical staining for AGRN in the muscularis mucosae (MM), which was absent in HP, TSA, TA, and other polyps. In contrast, histological evaluation showed only weak inter-observer agreement (kappa value = 0.493) in distinguishing SSLs.

Conclusions: Muscularis-mucosae-based AGRN immunostaining is a novel biomarker to distinguish SSL from HP, TSA and TA with a specificity of 97.1% and sensitivity of 98.9% and can assist in diagnosis of morphologically challenging colonic polyps.

Keywords

Agrin; Biomarker; Hyperplastic polyps; Immunohistochemistry; Sessile serrated lesions

Introduction

Colonic polyps differ in their risk for progression to cancer; consequently, recommendations for removal and further management are dependent on polyp classification (1). While tubular adenomas (TA) were traditionally considered to be the sole precursor lesions of

colorectal carcinoma (CRC), the recognition of the serrated polyp route to carcinoma has been an important milestone (2,3).

The World Health Organization (WHO) recognizes three categories of serrated polyps: traditional serrated adenomas (TSA), hyperplastic polyps (HP) and sessile serrated lesions (SSL) (4–8). HPs, predominantly arising in rectum and sigmoid colon, lack malignant potential and are by far the most common colonic polyps, accounting for approximately 80% of all serrated polyps (6). In contrast, SSLs, which do have malignant potential, are estimated to represent up to 20% of serrated polyps with a predilection for the right colon (9,10). TSAs also have malignant potential but represent only 1% of all serrated lesions (11).

While SSLs show abnormal basal crypt architecture, HPs lack these specific features (9). However, uncertainty exists regarding the minimum criteria for diagnosis of SSL and their distinction from HP. The number of abnormal crypts required has varied among guidelines from 1 to 3 SSL-like crypts (5–7). Furthermore, there is significant inter-observer variability as to what constitutes an abnormal crypt and poor specimen orientation and other biopsy artifacts accentuate the challenge (12–14). Indeed, substantial inter-observer variation among gastrointestinal (GI) pathologists remains and agreement among experts is moderate at best (13,15–20). These uncertainties often result in the inability to definitively distinguish SSL from HP. The diagnosis of SSL prompts more aggressive surveillance, generally 1–3 years, while a diagnosis of HP and small TAs (<10 mm) indicates less aggressive surveillance, every 5–10 years (5,18). Collectively, the evidence suggests a need to identify biomarkers for SSLs to guide appropriate management. Suggested biomarkers evaluated, MUC6, ANXA10 and HES1 (21–25) have not been rigorously tested nor are they used in routine practice.

To identify novel biomarkers for SSLs, we evaluated the expression of extracellular matrix (ECM) proteins upregulated in CRC (26). We investigated human colonic polyps and found differential AGRN positivity in the basal lamina (BL) of TA, TSA, HP and SSL whereas hamartomatous and mucosal prolapse polyps were negative. Importantly, SSL showed AGRN reactivity in the muscularis mucosae (MM) whereas other colonic polyps and controls were negative. Our study suggests that MM-based AGRN immunostaining represents a novel biomarker to differentiate SSL from HP, TSA and TA with a high specificity of 97.1% and sensitivity of 98.8%.

Materials and Methods

Patients and Samples

Patients were not involved in the study. Formalin-fixed, paraffin-embedded (FFPE) biopsies of normal colon and colonic lesions/polyps were identified from patients (between 2 and 89 years of age) undergoing colonoscopy between 2006 and 2019. Hematoxylin-eosin (H&E) slides were examined by two pathologists (VD, OHY) before inclusion in this study. We analyzed 408 colonic polyps, including hyperplastic polyps (HP; n=71), sessile serrated lesions (SSL) with dysplasia (n=23) and without dysplasia (n=166), traditional serrated adenomas (TSA; n=25), tubular adenomas (TA; n=64), dysplasia related to inflammatory bowel disease (IBD, n=29), sporadic adenomas arising in IBD (TA in IBD; n=9), mucosal

prolapse polyps (n=3). Colonic hamartomatous polyps (n=18) including juvenile polyps (n=10) and Peutz-Jeghers polyps (n=8) served as controls (Supplementary Fig. S9). The splenic flexure was used to differentiate left from right sided polyps; rectal polyps were categorized separately (see Supplementary Fig. S2 and Table S2). The SSL samples (n=189) included 132 (69.9%) polyps from the right colon, 49 (25.9%) from the left colon, and 8 (4.2%) rectal polyps. Among the 71 HP cases, 9 (12.7%) polyps were from the right colon, 32 (45.1%) from the left colon, and 30 (42.2%) from the rectum. HPs were further characterized as microvesicular HPs (MVHP; n= 38) and goblet cell HPs (GCHP; n= 33) by two pathologists (AN, VD, Supplementary Table S2) (27).

To define the serrated polyposis syndrome (SPS) we used criteria proposed by Syngal et al. (28). Briefly, diagnosis was based on the following criteria: (i) at least 5 serrated polyps proximal to the sigmoid colon with 2 of these being >10 mm; (ii) any number of serrated polyps proximal to the sigmoid colon in an individual who has a first-degree relative with serrated polyposis; or (iii) >20 serrated polyps of any size, distributed throughout the large intestine. Additional SPS patient samples were provided by the Department of Pathology, University of Utah (Salt Lake City, UT) (29).

Ethics

The study was approved by the Partners Institutional Review Board protocol (IRB #2017P000061) and the MIT IRB (Protocol #1408006568).

Majority-based pathology classification approach

Approach for majority agreement on fifty diagnostically challenging serrated polyps—We used a majority approach for the diagnosis of SSL. From our cohort of 408 colonic polyps and within the 100 cases validated by the 4 non-pathologists (see Supplementary Materials and Methods), two gastrointestinal (GI) pathologists identified fifty diagnostically challenging polyps with a low level of inter-observer agreement. Digital whole-slide H&E stained slides were evaluated by nine GI pathologists (DP, IB, ARM, LZ, QZ, RC, GL, OHY, VD) using the prior WHO guidelines (6). The pathologists were blinded to endoscopic size, endoscopic appearance, and location of the individual samples and classified the polyps into one of the following four categories: HP, SSL with or without dysplasia, TSA, or unclassified.

Approach for near-universal agreement on fifty SSLs—Given the existing lack of consensus among pathologists, we strove to identify fifty SSLs with near-universal agreement from within the cohort of 408 polyps. Among 65 additional polyps evaluated by nine GI pathologists (DP, IB, ARM, LZ, QZ, RC, GL, OHY, VD), all blinded to endoscopic size, endoscopic appearance, and location of the samples, we identified fifty SSLs with near-universal agreement.

Inter-observer variability among community pathologists—To assess the inter-observer variability on selected morphologically challenging polyps (see above), we further assessed fifty-five community-based pathologists. Based on representative images of H&E-stained slides from eight polyps the pathologists recorded their diagnosis using an audience

response system. Each pathologist was required to characterize the polyp into one of 5 categories: HP, SSL with or without dysplasia, TSA, TA or Rather not say (Supplementary Fig. S10).

Antibodies, Immunohistochemistry, Immunofluorescence, *In situ* hybridization and RNA-seq data analysis—Antibodies, standard techniques, RNA-seq data analysis and detailed information are provided in the Supplementary Materials and Methods and in Rickelt and Hynes (2018, ref. 30).

Results

Identification of extracellular matrix proteins in colonic polyps

To identify novel biomarkers to distinguish pre-invasive colorectal neoplasms we investigated the presence of 67 ECM proteins recently identified as increased in tumor samples from CRC patients (26). We used two parallel approaches to elucidate their value as potential biomarkers for detection of precancerous colonic lesions. First, we analyzed their expression levels in a publicly available RNA-seq dataset (29) on colonic polyp and control samples (Fig. 1). Second, we performed extensive immunohistochemical (IHC) screening on FFPE samples of diverse polyp types.

Gene expression analyses of sixty-five ECM molecules identified by proteomics (26) were performed using RNA-seq data (29) from forty-one colonic polyps including SSL (n=21), HP (n=10), TA (n=10) and colonic control samples (n=20). Most individual SSL samples clustered separately from the other polyp and control samples (Supplementary Fig. S1) clearly indicating that this class of polyps is distinct from others. To determine the most abundant ECM candidates upregulated in common colonic polyps, we performed differential-expression analysis comparing individual polyps to normal control tissues using cut-off selection criteria of log₂-fold change (FC) >1.0 and adjusted p value (adj) <0.05 (Fig. 2; Supplementary Table S1). This approach identified five ECM genes; agrin (*AGRN*), insulin-like growth factor binding protein, acid-labile subunit (*IGFALS*), S100 calcium-binding protein A11 (*S100A11*), serine peptidase inhibitor (*SERPINE2*) and TIMP metalloproteinase inhibitor 1 (*TIMP1*), all of which were upregulated in both SSLs and HPs (Figs. 1,2 and Supplementary Table S1) while their expression was lower in TAs.

In parallel, we screened and validated (on FFPE material) commercially available antibodies specific for CRC-associated ECM proteins (30). The combined IHC and transcriptomics approaches highlighted three ECM-associated proteins (*AGRN*, *SERPINE2* and *TIMP1*) with reliable antibodies, which were investigated further (Fig. 1).

AGRN and TIMP1 immunostaining in colorectal polyps

To investigate the potential diagnostic utility of *AGRN*, *SERPINE2* and *TIMP1* we performed IHC on a cohort of TAs, HPs, SSLs and TSAs (Supplementary Fig. S2) with antibodies to *AGRN*, *SERPINE2* and *TIMP1* (Supplementary Fig. S3). We detected strong staining for *AGRN* in the basal laminae (BL) of blood vessels and crypts of the polyps, and *TIMP1* in the cytoplasm of all polyp types investigated (Supplementary Figs. S2A and S3). In the normal colon, *AGRN* was only present in the BL of blood vessels and negative in the

normal colonic crypts. TIMP1 was also negative in the normal colon, however, neuroendocrine cells were positive for TIMP1, as previously described (31). In contrast, variable SERPINE2 cytoplasmic and ECM staining was noted in normal and lesional crypts (Supplementary Fig. S3). Given the lack of a consistent and clear differential reactivity with TIMP1 and SERPINE2 in normal versus polyp tissue, we elected to evaluate AGRN further.

Differential localization of AGRN as a biomarker of SSLs

In total, we evaluated four-hundred-eight colonic polyps representing the following pathologic diagnoses: HP (n=71), SSL with (n=23) and without (n=166) dysplasia, TSA (n=25), TA (n=64), colonic hamartomatous (n=18) and mucosal prolapse polyps (n=3) (Supplementary Figs. S2B–E, S9, Table S2). We found positive AGRN staining of the BL in all colonic lesions, whereas hamartomatous, mucosal prolapse polyps and normal colon mucosa were negative. However, differential patterns of BL localization were noted (Fig. 3); a top-heavy pattern of AGRN positivity was consistently observed in TAs, whereas in TSAs the BL reactivity was uniformly distributed along the length of the colonic crypt. In contrast, in SSL and HP samples, AGRN BL positivity was more prominent in the basal crypts, with weaker staining consistently seen in HPs.

To examine further the biological relevance of these findings, we assessed the BL AGRN positivity in a range of mouse models with conventional adenoma (Supplementary Fig. S4A–D) and those with serrated-like (Supplementary Fig. S4E) morphology. In concordance with the human samples, AGRN immunostaining with two anti-AGRN antibodies confirmed the consistent presence of this protein in the BL of murine colonic polyps while the BL of adjacent normal colonic mucosa was negative (Supplementary Fig. S4). More importantly, the BL distribution patterns of AGRN in the mouse models paralleled the human data (Supplementary Fig. S4). Collectively, the genetically defined mouse models of colonic polyps recapitulate the distribution patterns of AGRN seen in human TAs and TSAs.

AGRN is expressed in epithelial cells and deposited into the muscularis mucosae in SSL

Strikingly, we also noted the selective presence of muscularis mucosa (MM)-based AGRN in human SSLs with and without dysplasia but not in other polyps (Fig. 3 and Supplementary Fig. S3). IHC and confocal immunofluorescence microscopy on consecutive sections of SSLs demonstrated AGRN co-localization with SMA and DES (both muscle-specific markers) in the MM (Fig. 4A–D); AGRN was localized to the upper half of the MM (Fig. 4D). *AGRNM* mRNA expression was predominantly localized to crypt-base cells with stronger expression in SSL as compared with HP, TA and TSA (Supplementary Fig. S5A–H), while the adjacent MM of SSLs was negative (Supplementary Fig. S5E,F, arrowheads). Collectively, these results suggest that *AGRNM*, expressed by colonic epithelial cells, is deposited in the MM in SSLs.

AGRN positivity of the muscularis mucosae as a biomarker for SSLs

To investigate further the utility of MM-based AGRN reactivity in diagnosing SSLs we investigated twelve anti-AGRN antibodies on human FFPE samples (30), however, we identified only one additional reliable anti-AGRN antibody. Both anti-AGRN antibodies

showed similar staining patterns (Supplementary Fig. S5I–L); and selectively stained the MM of SSL.

We next identified fifty SSLs with near-universal diagnostic agreement among nine expert GI pathologists. MM-based AGRN reactivity was noted in all samples (data not shown). In addition, we tested 18 SSL polyps from ten serrated polyposis syndrome (SPS) patients (a single polyp in two patients and 2 polyps in eight patients) and all showed a strong MM-based positivity for AGRN (Supplementary Fig. S6).

To control for bias, since experienced pathologists can recognize a SSL without an AGRN stain, four non-pathologists were asked to blindly validate the MM-positivity for AGRN on one-hundred samples (Supplementary Fig. S7A, Supplementary Table S2), including all cases used in the validation cohort (see below). Notably, there was complete agreement with regard to MM-based reactivity among the four observers (Supplementary Fig. S7B), validating the robustness of this criterion.

Finally, we selected fifty diagnostically challenging HPs, SSLs and TSAs (Supplementary Table S2) and compared the results of AGRN staining with the evaluation performed by nine GI pathologists on H&E sections (see Materials and Methods). In total, samples #1-46 were scored (Fig. 5A); four samples had to be excluded (samples #47-50, Supplementary Fig. S7C). Complete concordance among the pathologists was achieved in only 13 cases (26%), kappa value = 0.493, indicating only weak overall agreement (Fig. 5A, green). The principal source of disagreement lay in the distinction of HP (Fig. 5A, grey) from SSL (Fig. 5A, orange). Based on majority opinion (at least 5 of 9 pathologists) the polyps were classified as follows: 25 SSLs with or without dysplasia, 14 HPs and 4 TSAs (Fig. 5A, purple). Three samples did not yield a majority and the opinions were widely divergent. The MM staining for AGRN was identified (Fig. 5A, red) in 24 of the 25 cases diagnosed as SSL. In contrast, 19 cases that lacked a majority opinion diagnosis of SSL were negative for AGRN in the MM (Fig. 5A, blue). In the three cases lacking a majority opinion (arrows in Fig. 5A), the results differed from those noted above and are illustrated in Fig. 5B–G. Although SSL represented the majority (5 of 9) opinion in case #25, no AGRN reactivity was noted (Fig. 5B,C). Two cases #26 and #27 both showed MM-based AGRN reactivity, however, for #26 no majority opinion was reached and #27 majority (5/9) opinion was HP, although both cases showed contiguous crypts with basal crypt dilatation and basal serrations (Fig. 5D–G). Finally, we also tested fifty-five community pathologists by showing them representative images of H&E slides from eight cases and observed a virtually similar level of inter-observer agreement (Supplementary Fig. S10).

Collectively, for all samples investigated in this study, the majority reads of the AGRN staining used for final calculations were as follows: MM-based AGRN positive staining in 186/188 SSLs with (23/23) and without (163/165) dysplasia, 5/68 HPs, 0/64 TA, 0/25 TSA and 0/21 hamartomatous and mucosal prolapse polyps (Supplementary Figs. S7D, S9, Supplementary Table S2). AGRN reactivity also assisted in differentiating TA from SSL with dysplasia when the continuity between the SSL and dysplastic component was lost due to fragmentation of the specimen (Supplementary Fig. S8). Additionally, loss of BL-based AGRN reactivity was noted in the dysplastic portion of SSL (Supplementary Fig. S8D,I,J).

In summary, these observations suggest that the MM-based AGRN stain could help to assist in identifying dysplasia arising within SSL in cases in which the SSL component is poorly represented. Given that SSL with dysplasia are more likely to progress to cancer it is important to accurately diagnose a dysplastic component of serrated polyps.

Finally, samples of both subtypes of HPs (MVHP, n=37 and GCHP, n=32) and 9 large HPs (>4.5 mm) were negative for MM-based AGRN (see also Supplementary Table S2).

Power analyses ensured sufficient precision of our estimation. Based on sensitivity and specificity, a 95% confidence interval as narrow as 7% was calculated. Collectively, this provides a reliable estimation of MM-based AGRN as a diagnostic marker for SSLs with sensitivity of 98.9% and specificity of 97.1% (Supplementary Fig. S7D).

RNA-seq-profiled colonic polyps corroborate the biomarker capability of AGRN immunoreactivity

We finally sought to validate our AGRN immunohistochemical findings on the cohort of polyps from the RNA-seq dataset (29) (Figs. 1, 2; Supplementary Fig. S1). Many SSL (n=14) and a few HP samples (n=4) showed higher *AGRN* expression, most other polyps and controls showed lower *AGRN* expression (Fig. 6A). We evaluated 10 SSL and 5 HP for MM-based AGRN reactivity (Fig. 6). Blinded evaluation identified 9 out of 10 SSL to be AGRN-positive (data not shown). The majority of these SSLs also showed elevated *AGRN* expression and formed a distinct cluster (Supplementary Fig. S1), three samples (#14, 18, 20) did not conform with our hypothesis (Fig. 6, Supplementary Fig. S1, see figure legend for details).

Finally, in agreement with our prior observation (see above) all 12 samples from SPS patients in this cohort (29) clustered together and showed high *AGRN* expression (Fig. 6A, Supplementary Fig. S1). In addition, of these samples all six polyps that we stained were positive for MM-based AGRN reactivity (Fig. 6A).

Discussion

SSLs have a risk of malignant progression and require accelerated screening for CRC. However, in routine clinical practice, histopathological characterization of SSLs has met significant challenges; overlapping histological features with HPs, poor biopsy orientation, resulting in moderate-to-poor inter-observer agreement among pathologists (15–17,19,20). A biomarker specific for SSLs would clearly help pathologists and allow evidence-based surveillance of serrated polyps, and the results presented here suggest that MM-based AGRN reactivity could serve as such an objective marker for SSL.

There have been several prior attempts to identify SSL-specific biomarkers. Caruso et al (32) performed a gene-array study and compared SSLs to TAs and controls. SSLs showed upregulation of *CTSE* and *TFF1*. ANXA10 was identified in SSLs as compared to HPs and validated by IHC as a potential diagnostic marker with a sensitivity of 73% and specificity of 95% in the diagnosis of SSL (24). MUC6 was found to be expressed in SSL but not in HP (21), however, subsequent studies revealed a relatively low sensitivity (22) and lack of

specificity for SSL (23). In addition, Delker et al (33) examined gene expression to discriminate between SSLs and HPs and found unique staining patterns for the cell junction protein VSIG1 and MUC17 in SSLs. Finally, the loss of the transcription factor HES1, was observed in the majority of SSLs compared to normal expression in HPs (25), however, TA and also TSA showed variable staining for HES1, diminishing its value as a marker of SSL. Notably, none of these potential biomarkers is currently applied in routine clinical practice.

ECM proteins play an important role in the initiation and progression of colon carcinomas, often related to poor prognosis (34,35). The diagnostic relevance of ECM proteins in CRC and their applicability to distinguish individual colonic polyps so far remains untested. Our results demonstrate the differential expression of several ECM genes among colonic polyps and show that *AGRN*, *SERPINE2* and *TIMP1* are overexpressed in SSL and HP samples (Figs. 1, 2; Supplementary Figs. S1, S3). Colonic polyps show distinct patterns of AGRN deposition in BL. Importantly, MM-based AGRN reactivity was restricted to SSLs (Figs. 3, 4, Supplementary Figs. S3, S5). We confirmed this finding by evaluating a cohort of fifty diagnostically challenging colonic polyps with only weak agreement among GI pathologists. Virtually all cases with a majority read of SSL showed MM-based AGRN reactivity, while non-SSL polyps lacked AGRN reactivity in the MM. Another strong validation of the diagnostic value of MM-based AGRN reactivity is the near-universal presence of MM-based AGRN in SPS patients.

The ECM molecule AGRN, a large multidomain heparan sulfate proteoglycan, is abundantly expressed in developing brain and in virtually all BL of developing organs. Functionally, AGRN aids in the formation of neuromuscular junctions and acetylcholine receptor clustering in the central nervous system (36). The non-neuronal functions of AGRN are only poorly understood. Although little is known about AGRN in carcinogenesis, in recent years, a tumor-promoting role has been reported for several cancer types, including hepatocellular and cholangiocellular carcinoma (37,38), prostate cancer (39) and oral squamous cell carcinoma (40). Functional studies have shown that AGRN is involved in proliferation, migration and invasion of liver cancer cells by regulating focal adhesion integrity and to relay mechanosensitive signals into cells to regulate YAP activity to promote tumorigenesis (41,42). In contrast, the role of AGRN in normal colorectal mucosa, colonic polyps and CRC has not been investigated extensively. By tissue secretome profiling, AGRN was one of seventy-six potential CRC protein biomarkers that may facilitate blood- or stool-based assay development to support clinical management of CRC (43). Another analysis of the extracellular proteome of CRC cells identified elevated levels of the C-terminal fragment of AGRN (44), however, its suitability as a biomarker has not yet been assessed.

Although expressed by colonic epithelial cells, AGRN is specifically localized in the MM of SSL. AGRN interacts with several other ECM proteins and a variety of growth factors. In addition, earlier studies have shown AGRN to bind cell-surface adhesion receptors, such as NCAM, integrins, α -dystroglycan and muscle-specific kinase (36). The determination of whether any of these proteins co-localize at the MM of SSLs and interact with AGRN will therefore be of interest.

In conclusion, our study supports the use of MM-based reactivity for AGRN as a novel biomarker to differentiate SSL from HP. Our study uses a consensus opinion as the 'comparison standard', and MM-based AGRN expression compared very favorably with this standard, outperforming H&E-based diagnosis by expert pathologists in a set of challenging SSL; whereas in a set of straightforward SSL the results of AGRN staining were concordant with virtually all pathologists. Finally, MM-based AGRN was also universally detected in SPS syndrome. Thus, MM-based immunostaining for AGRN may enable more accurate diagnosis of patients with SSLs and assist with morphologically challenging cases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

| | |
|-------------|----------------------|
| AGRN | agrin |
| BL | basal lamina |
| CRC | colon carcinoma |
| CL | control left |
| CR | control right |
| CRC | colorectal cancer |
| DES | desmin |
| ECM | extracellular matrix |

| | |
|-----------------|--|
| FFPE | formaldehyde-fixed and paraffin-embedded |
| GCHP | goblet cell hyperplastic polyp |
| GI | gastrointestinal |
| H&E | hematoxylin-eosin |
| HIER | heat-induced epitope-retrieval |
| HP | hyperplastic polyp |
| IBD | inflammatory bowel disease |
| IGFALS | insulin-like growth factor binding protein acid labile subunit |
| IHC | immunohistochemistry |
| ISH | <i>in situ</i> hybridization |
| JPS | juvenile polyps |
| mAbs | monoclonal antibodies |
| MM | muscularis mucosae |
| MVHP | microvesicular hyperplastic polyp |
| PJP | Peutz-Jeghers polyps |
| RNA-seq | RNA sequencing |
| S100A11 | S100 calcium-binding protein A11 |
| SERPINE2 | serine peptidase inhibitor, clade E, member 2 |
| SMA | smooth-muscle actin |
| SPS | serrated polyposis syndrome |
| SSL | sessile serrated lesions |
| TA | tubular adenoma |
| TIMP1 | TIMP metalloproteinase inhibitor 1 |
| TSA | traditional serrated adenoma |
| VIM | vimentin |

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Translational Relevance

Sessile serrated lesions (SSL) are precursors of colon carcinoma. Their distinction from other polyps with better prognoses, most notably hyperplastic polyps (HPs), often presents a significant diagnostic challenge and histological evaluation shows only weak inter-observer agreement among experts. We identified the extracellular matrix protein agrin in the muscularis-mucosae (MM) of SSL biopsies but not in other polyps or normal colonic tissue. Immunohistochemical staining of the MM for agrin presents a novel biomarker with high specificity and sensitivity and markedly improves the discrimination among polyp subtypes. Immunostaining for agrin in the muscularis mucosae enables pathologists towards more accurate diagnoses of patients with SSLs, can assist with morphologically challenging cases and may allow evidence-based surveillance of serrated polyps.

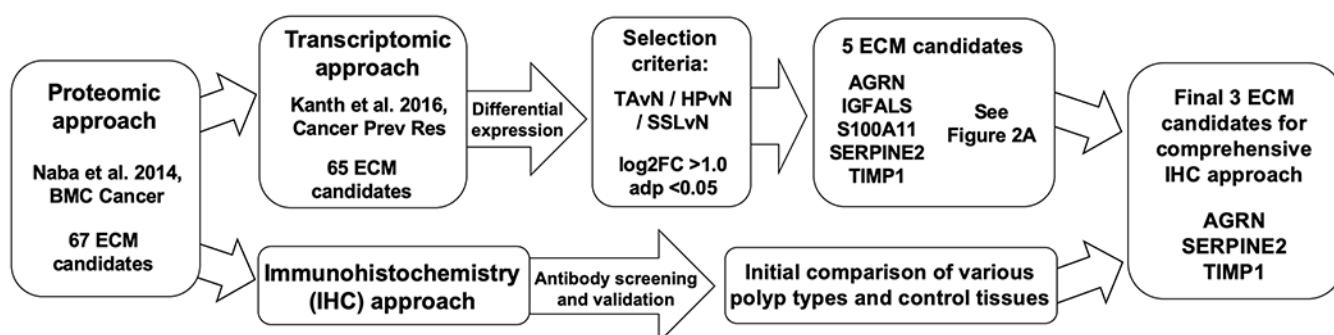


Figure 1. Summary of selection of biomarker candidates.

Strategies to identify candidate extracellular matrix (ECM) genes and proteins involved in colonic polyp development. ECM biomarker proteins upregulated in colorectal cancer (CRC) and CRC-derived liver metastasis (Naba et al. 2014, ref. 26) were investigated for their presence in colonic polyps using two independent approaches; [1] Screening by immunohistochemistry (IHC) using reliable antibodies applicable to FFPE patient samples and [2] differential expression analyses of publicly available patient RNA-seq datasets (Kanth et al. 2016, ref. 29). These approaches identified three relevant ECM proteins: AGRN, TIMP1 and SERPINE2.

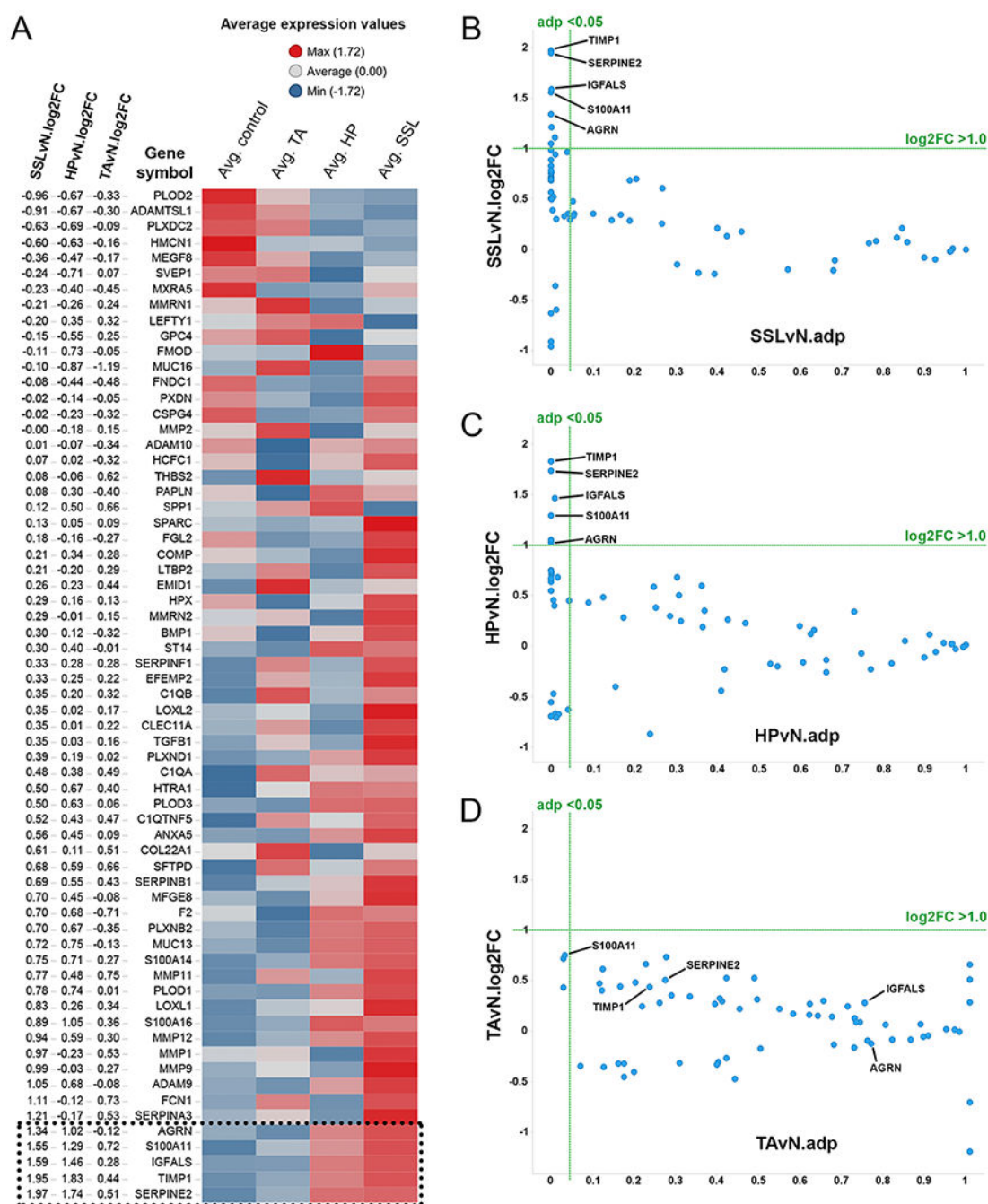


Figure 2. Differential expression analysis to identify candidate extracellular matrix genes in human colon polyps.

Differential expression analysis of sixty-five genes encoding ECM proteins previously identified to be upregulated in patients with colorectal cancer (26) using RNA-seq data (29) from a variety of colonic polyps including sessile serrated lesions (SSL; n=21), hyperplastic polyp (HP; n=10), tubular adenoma (TA; n=10) and normal colon controls (N; n=20).

A) Row-centered average expression values for all three polyp types and normal controls are plotted in the heatmap. The rows are rank-ordered according to SSLvN log2 fold changes (log2FC) as shown in the first three columns. Five ECM genes which meet the threshold

[log2FC >1.0 and adjusted p value (adp) <0.05] in SSL and HP samples are indicated at the bottom of the heatmap. See Supplementary Fig. S1 for gene expression data of the individual samples.

B-D) Scatterplots showing the distribution of expression levels of the sixty-five ECM-protein genes and selection criteria; log2FC >1.0 and adp <0.05 (green lines) among SSL (**B**), HP (**C**) and TA (**D**) samples analyzed. Highlighted are the five genes (*AGRN*, *IGFALS*, *S100A11*, *SERPINE2*, *TIMPI*) which meet the selection threshold and are overexpressed in SSLs and HPs but not TAs.

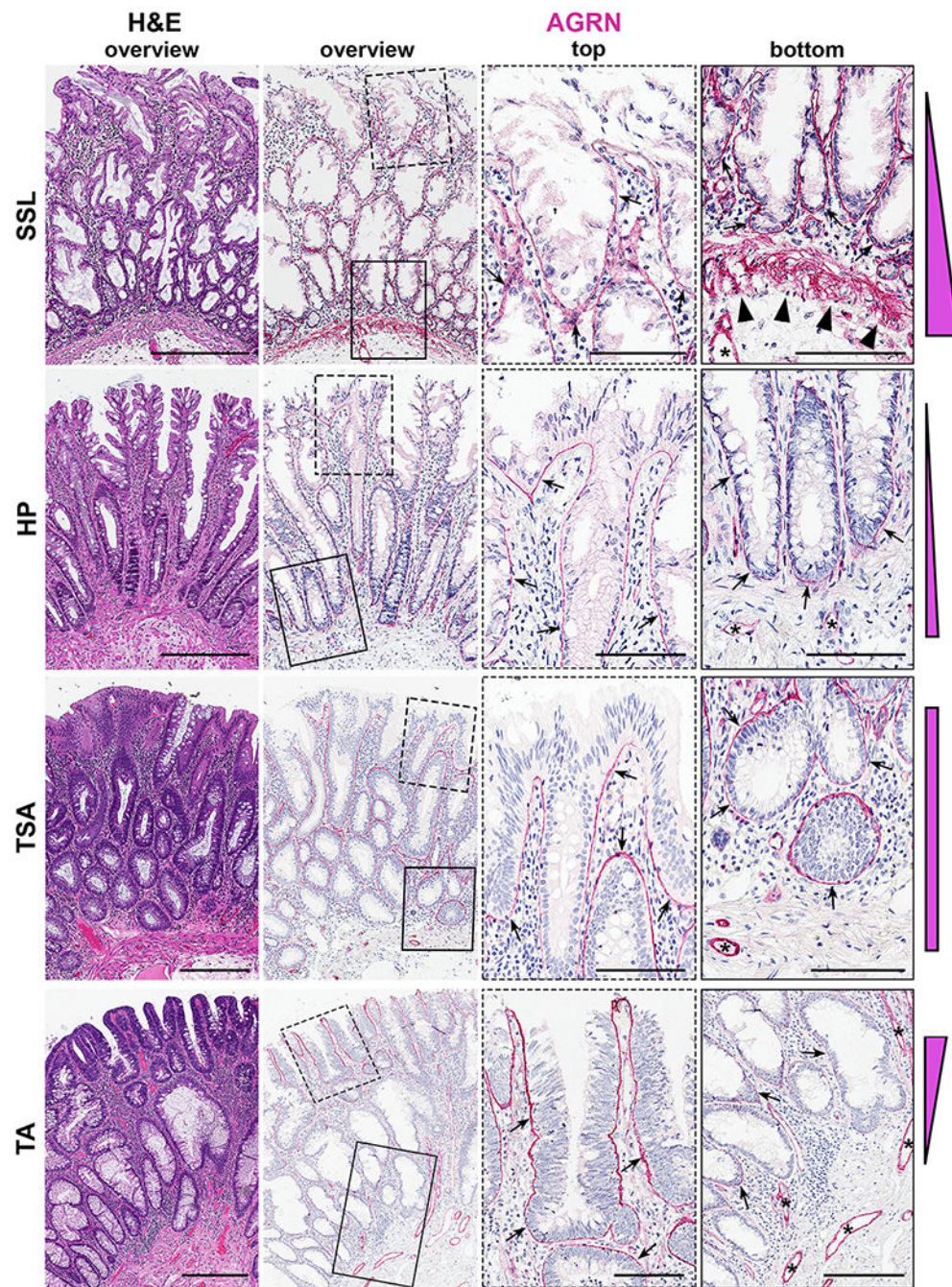


Figure 3. Differential localization of AGRN as a biomarker to distinguish colorectal polyps. Representative H&E and agrin (AGRN) IHC images of colonic polyps. Presented are overview images (left two columns) and enlarged images for AGRN IHC (boxed areas) from the top and the bottom of the crypt (right two columns) for the individual polyp types. Note the positive stain in the basal lamina (BL) of all blood vessels (*) and the differential localization patterns of AGRN in the BL (indicated by arrows) of the different types of polyps (also presented schematically at the right edge of each polyp panel). AGRN reactivity is consistently observed as follows: SSLs and HPs (basal crypt predominance); TSAs (top-

and bottom-high); TAs (top-high-to-bottom-low). Also note the presence of AGRN in the muscularis mucosa (MM) exclusively in SSL (arrowheads). Scale bars: 300 μm (overviews) and 100 μm (magnifications)

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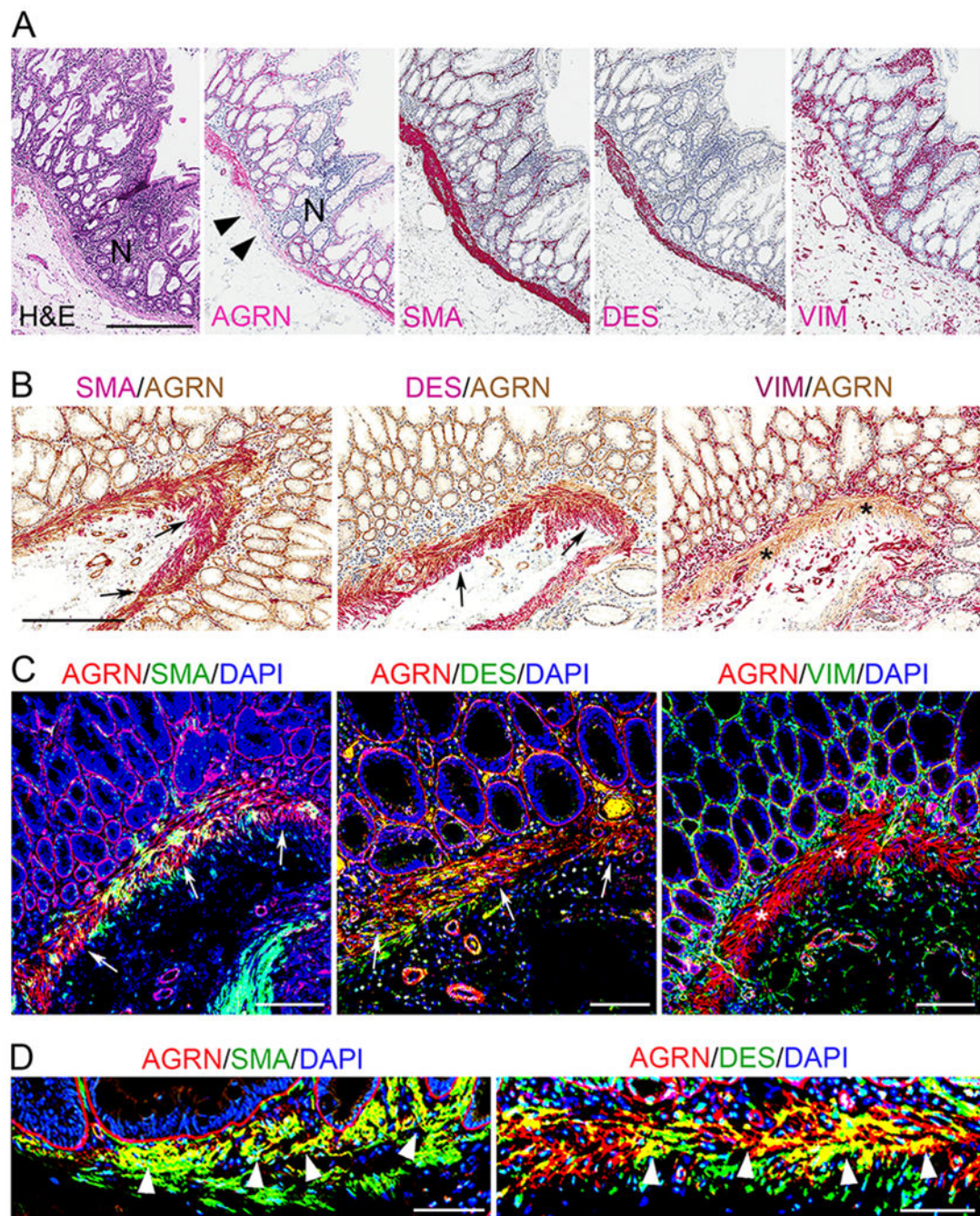


Figure 4. AGRN reactivity in the muscularis mucosae of sessile serrated lesions

A) Representative H&E and IHC images of parallel sections of SSL comparing AGRN reactivity to smooth-muscle actin (SMA), desmin (DES) and vimentin (VIM). Scale bar: 300 μ m

B) IHC images of immunostains for AGRN (brown), SMA, DES and VIM (red) performed on consecutive sections. AGRN colocalizes with SMA and DES but not with VIM. Scale bar: 300 μ m

C and D) Confocal immunofluorescence microscopy of SSL tissue sections, comparing the localization of AGRN (red) with that of SMA, DES and VIM (all in green); yellow signal shows colocalization. Cell nuclei are shown in blue. Scale bars: 100 μm (**C**), 50 μm (**D**) AGRN is localized to MM, which is also positive for SMA and DES (**A-D**) but negative for VIM (*; **B and C**). Arrows in **B and C** mark the co-localization of AGRN with SMA and DES (color overlay in **B**, and yellow merged color in **C and D**). Also note, AGRN is exclusively present in the MM adjacent to the abnormal crypts of SSL and ends, often abruptly, in the adjacent normal (N) crypts (black arrowheads in **A**). Magnified images reveal that AGRN mainly localizes to the upper half of the MM (white arrowheads in **D**).

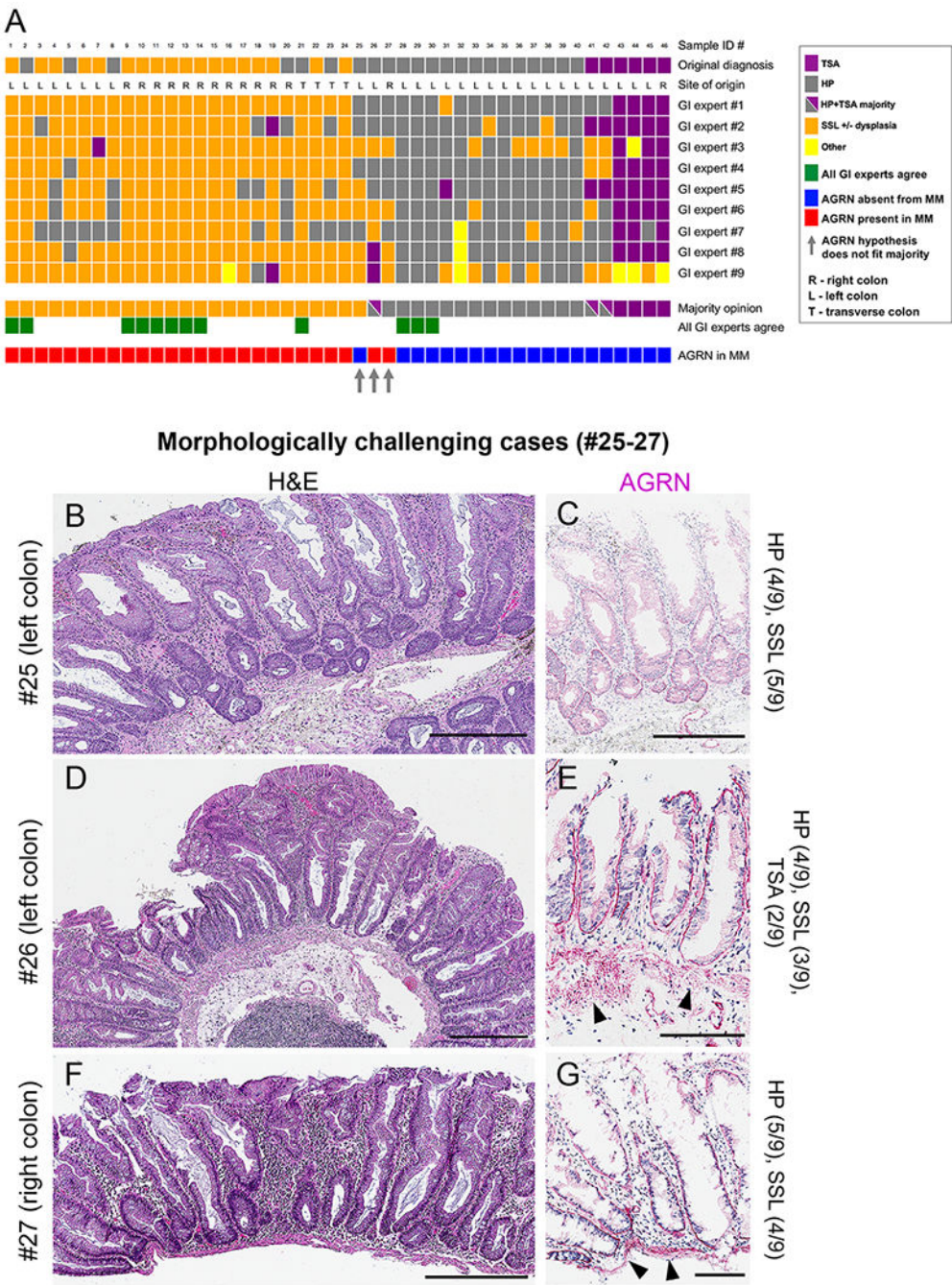


Figure 5. Majority-based polyp validation among expert gastrointestinal pathologists. Nine experts in gastrointestinal (GI) pathology classified fifty diagnostically challenging polyps by H&E into: 1) TSA, 2) HP, 3) SSL) +/- dysplasia and 4) other, according to previous WHO criteria (see text for details). A) Presented are the results of forty-six of these cases (for the four samples not shown see Supplementary Fig. S7). Indicated are the original diagnoses, the site of polyp origin and the individual GI expert opinions. For each case, the majority pathologist's opinion is compared to the corresponding MM-positivity for AGRN as scored by 4 non-pathologists

(Supplementary Fig. S7A). Note the diagnostic variability among the individual GI experts as compared with the high concurrence of AGRN-positive MM staining with SSL (majority opinion) whereas most HPs and TSAs are negative.

B-G) H&E (**B,D,F**) and corresponding AGRN IHC (**C,E,G**) images of three morphologically challenging cases (#25-27) as indicated by the three **grey arrows in (A)** in which the AGRN-positive stain of MM differs from the majority GI expert opinion, although those opinions were widely divergent. (**C**) Note the absence of AGRN from the MM of sample #25 (~1.8x4.8mm, left colon; 4/9 GI experts classified this as HP and 5/9 as SSL). (**E and G**) Note AGRN-positive MM (arrowheads) in samples #26 (~2.1x1.6mm, left colon); 4/9 GI experts classified this as HP, 3 as SSL and 2 as TSA and #27 (~0.55x1.8mm, right colon); 5/9 GI experts classified this as HP and 4 as SSL). Notably, these polyps show 2 contiguous crypts with basal dilatation, meeting the WHO definition of SSLs. See also Supplementary Table S2 for individual samples. Scale bars: 300 μ m (**B,D,F**), 100 μ m (**C,E,G**)

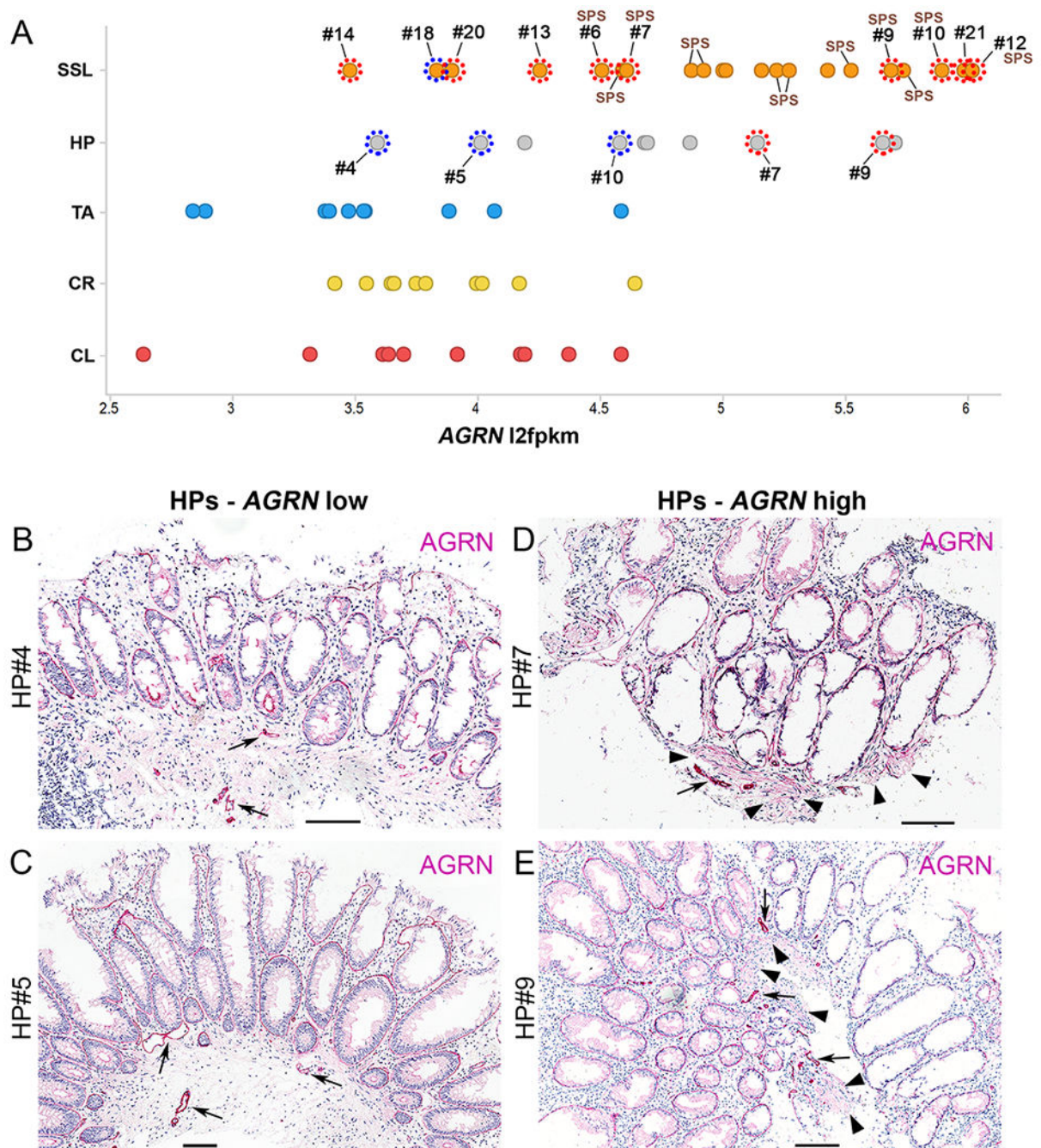


Figure 6. *AGRN* IHC validation on colonic polyp samples previously analyzed by RNA-seq.

A) Scatter plot of *AGRN* mRNA expression values for SSL (n=21), HP (n=10), TA (n=10) and colon controls (CL, control left, n=10; CR, control right, n=10) using publicly available RNA-seq data (29) (see Supplementary Fig. S1). Expression values of *AGRN* (log2 fragments per kilobase million) for the individual polyps and controls are indicated and diagnosis highlighted by a colored dot: orange (SSL), grey (HP), blue (TA), yellow (CR), red (CL). Note that many SSLs and a few HPs show high *AGRN* values (above 5) whereas all other samples show lower *AGRN* expression. Examples of SSL (n=10) and HP (n=5) for

which sections were stained for AGRN are indicated by dotted circles; positive (red dotted circles) or negative (blue dotted circles) for MM-based AGRN reactivity. Note that the two designated HP samples showing high *AGRN* expression levels also scored positive for MM-based AGRN, whereas those with lower overall *AGRN* expression were also negative for AGRN MM staining. Most SSL samples scored positive for MM-based AGRN staining. SSL samples from serrated polyposis syndrome (SPS) patients are indicated.

B-E) AGRN IHC images of sections from four HPs samples from the RNA-seq dataset (29) presented in **A**. Shown are sections of HP samples in which the *AGRN* expression values are low [HP#4 (**B**), HP#5 (**C**)] or high [HP#7 (**D**), HP#9 (**E**)]. Note also in two of five HP samples the additional positivity for AGRN in the MM (HP#7, HP#9, arrowheads in **D** and **E**). Notably, these two cases cluster with the majority of SSLs whereas HPs#4 and HP#5 (negative for MM-based AGRN) cluster separately (Supplementary Fig. S1). Scale bars: 100 μ m