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Germline Mutations in Oncogene-Induced Senescence Pathways are Associated with Multiple Sessile Serrated Adenomas

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Abstract

Background & Aims—Little is known about the genetic factors that contribute to development of sessile serrated adenomas (SSAs). SSAs contain somatic mutations in *BRAF* or *KRAS* early in development. However, evidence from humans and mouse models indicates that these mutations result in oncogene-induced senescence (OIS) of intestinal crypt cells. Progression to serrated neoplasia requires cells to escape OIS, via inactivation of tumor suppressor pathways. We investigated whether individuals with multiple SSAs carry germline loss-of-function mutations (nonsense and splice-site) in genes that regulate OIS – the p16–Rb and ATM–ATR DNA damage response pathways.

Methods—Through bioinformatic analysis of the literature, we identified a set of genes that function at main nodes of the p16–Rb and ATM–ATR DNA damage response pathways. We performed whole-exome sequencing of 20 unrelated individuals with multiple SSAs; most had features of serrated polyposis. We compared sequences with those from 4300 individuals, matched for ethnicity (controls). We also used an integrative genomics approach to identify additional genes involved in senescence mechanisms.

Results—We identified mutations in genes that regulate senescence (*ATM*, *PIF1*, *TELO2*, *XAF1*, and *RBL1*) in 5/20 individuals with multiple SSAs (odds ratio [OR]=3.0; 95% confidence interval [CI], 0.9–8.9; *P*=.04). In 2 individuals, we found nonsense mutations in *RNF43*, indicating that it is also associated with multiple serrated polyps (OR=460; 95% CI, 23.1– 16384; *P*=6.8×10^{−5}). In

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knockdown experiments with pancreatic duct cells exposed to ultraviolet light, RNF43 appeared to function as a regulator of ATM–ATR DNA damage response.

Conclusions—We associated germline loss-of-function variants in genes that regulate senescence pathways with the development of multiple SSAs. We identified *RNF43* as a regulator of the DNA damage response, and associated nonsense variants in this gene with high risk of developing SSAs.

Keywords

serrated polyposis syndrome; hereditary colon cancer; sessile serrated polyp; RNF43

Background and Aims

Sessile serrated adenomas (SSA) are a newly recognized precursor lesion to colorectal cancer. Found in 2% of average-risk individuals undergoing their first screening colonoscopy, SSAs are believed to give rise to sporadic microsatellite instability-high (MSI-H) colon cancers, as well as *BRAF*-mutated microsatellite stable (MSS) colon cancers.^{1, 2} Despite their relative infrequency, serrated lesions are estimated to be responsible for approximately 20–35% of all colon cancers.^{3, 4} In contrast to tubular adenomas, SSAs are not dependent on mutations in the *APC*-beta catenin axis, but instead exhibit somatic mutations in *BRAF*, or less commonly *KRAS*, early in their development.^{4–6} To determine the presently unknown genetic susceptibility for this lesion, we chose to study individuals who develop multiple SSAs, a hallmark feature of serrated polyposis syndrome.

Although there is no clear consensus on the clinical definition of the serrated polyposis syndrome, its prevalence has been estimated to be as high as 1:3000 in populations primarily composed of European ancestry.⁷ Affected individuals possess as high as a 54% lifetime risk of colon cancer, and may display strong personal or family histories of extracolonic cancers.^{8, 9} Despite the broad phenotypic variability observed with this syndrome, epidemiological evidence demonstrates an underlying genetic risk. First degree relatives have a 32% risk of developing multiple serrated polyps, as well as a five-fold increased risk of colon cancer.^{10, 11} More recently, an increased risk of pancreatic cancer has also been observed.¹² Attempts to model the genetics of this syndrome as monogenic among unrelated individuals, or as Mendelian through linkage analyses, have not been successful.^{13, 14} The genetic basis for this disorder remains undefined, with the exception that mutations in *MUTYH* have been observed in only a small minority of cases that present with concomitant tubular adenomas.¹⁵

Given this variable clinical presentation within an extreme phenotype and likely underlying genetic complexity and heterogeneity, we hypothesized that high-risk variants may be localized to specific pathways instead of a single gene. Whereas mice with inactivating mutations in *APC* rapidly develop tubular adenomas, *BRAF* or *KRAS* mutations that are associated with serrated polyps are alone insufficient to provoke intestinal tumorigenesis.^{16, 17} After a brief period of hyperproliferation, crypt cells undergo growth arrest due to metabolic and replicative stress, a process termed oncogene-induced senescence (OIS). Additional mutational events are required for neoplastic progression.¹⁸ Accordingly, engineered bypass of OIS at the genetic level quickly recapitulates the serrated neoplasia phenotype found in humans.¹⁷ In the human colon, OIS has been demonstrated to be primarily mediated by the p16-RB and ATM-ATR-mediated DNA damage response (DDR) pathways.^{19, 20} Since bypass of OIS is fundamental to SSA progression, we sought to determine whether individuals with multiple SSAs are enriched with germline, strong loss-of-function mutations (nonsense and splice-site) within either of these two pathways.

Materials and Methods

Ethics Statement and Study Recruitment

This study was approved by the Institutional Review Board of the Massachusetts General Hospital (MGH) under Protocol #2011P001161. Written informed consent was obtained from all study participants. Twenty unrelated study participants were recruited from clinics in the Gastroenterology Division of MGH.

Given our focus on SSAs specifically and not serrated polyps in general, we enrolled individuals diagnosed with multiple SSAs who fulfilled a set of criteria that were modified from the World Health Organization criteria for serrated polyposis in order to define a robust genotype-phenotype relationship for sessile serrated adenomas (Table 1).²¹ We exclusively counted SSAs regardless of size toward multiplicity thresholds despite the existence of other types of serrated polyps in study participants. Family history of serrated polyposis was expanded to include a family history of colorectal cancer given the strong associated genetic risk and the absence of SSAs from older pathology reports.²² Participants were excluded if they were previously diagnosed with a known polyposis syndrome through genetic testing. However, no individuals were excluded as a result of a positive genetic test.

Given that all study participants were of complete (95%) or partial (5%) European descent, we used the exomes of 4300 European-Americans from the NHLBI Exome Sequencing Project as genetic controls.²³ No polyp or cancer histories are available for these individuals, and were assumed to be negative to bias the study toward the null hypothesis.

Gene Set Construction

Prospectively, we defined a gene set for the p16-Rb and ATM-ATR DDR pathways for which strong LoF mutations could impair OIS. Core components of the p16-Rb axis whose loss would result in impairment were previously defined as *CDKN2A*, *CDKN2B*, *CDKN2C*, *CDKN2D*, *CDKN1A*, *CDKN1B*, *RB1*, *RBL1*, and *RBL2*.²⁴ Given the variable impact according to cell type of the numerous downstream effectors of the ATM-ATR-mediated DDR on OIS, we defined the critical nodes for this pathway as all genes whose loss results in diminished activation or function of DNA checkpoint kinases: ATM, ATR, Chk1, or Chk2. While p53 has been shown to be a critical downstream effector of senescence in other cell types, multiple animal studies have demonstrated that biallelic loss of p53 fails to increase intestinal polyp multiplicity, or accelerate progression of early stage conventional adenomas or serrated polyps.^{17, 18, 25} As such, we did not incorporate p53 into our analysis.

A python script was devised to extract all 19,049 HGNC gene symbols of protein coding genes and all associated one-word gene synonyms from the ExPASy GPSDB Gene/Protein Synonyms finder (<http://gpsdb.expasy.org/#>). Subsequently, this script employed the e-utilities function of Pubmed (<http://www.ncbi.nlm.nih.gov/books/NBK25500/>) to cross-reference each gene name and its synonyms sequentially against: “ATM”, “ATR”, “Chk1”, and “Chk2”. All search terms were coded as text words. This search algorithm yielded 313,747 Pubmed IDs last scanned as of December 11, 2012. Each title, abstract, and/or publication was manually reviewed. This analysis yielded 224 genes whose loss impaired activation or function of ATM, ATR, Chk1, or Chk2 (Supplementary Table 1). In addition to the 9 genes from the p16-RB pathway, the final gene set consisted of 233 genes for genes relevant to OIS pathways in the colon.

Genes containing cancer-related GWAS loci ($P < 1.0 \times 10^{-5}$) were obtained from the NHGRI Catalog of Genome-wide Association Studies (<http://www.genome.gov/gwastudies/>), which assigned loci to the closest gene if applicable. The search terms “cancer”, “tumor”, “leukemia”, “lymphoma”, “sarcoma”, “melanoma”, “myeloma”,

“rhabdomyosarcoma”, and “schwannoma” were used to compile a list of 555 genes containing such loci (Supplementary Table 2).

Exome Sequencing

Genomic DNA was isolated from peripheral blood, or mouthwash samples if study participants harbored active hematopoietic neoplasms (Qiamp, Qiagen). Exome sequencing was performed by the Orogenetics Corporation (Norcross, GA). Illumina libraries were made from qualified fragmented genomic DNA (NEBNext reagents, New England Biolabs) and the resulting libraries were subjected to exome enrichment using NimbleGen SeqCap EZ Human Exome Library v2.0 (Roche NimbleGen, Inc.) or SureSelectXT Human All Exon v4 (Agilent Technologies, Inc.) following the manufacturer's instructions. The samples were sequenced on the Illumina HiSeq2000 platform which generated paired-end reads of 100 nucleotides. Samples were sequenced to a mean depth greater than 50× (Supplementary Table 3).

The exome sequencing of 4300 individuals comprising the European-American cohort of ESP6500 was performed as previously described.²³ Of note, sequencing was performed at a mean depth of ~111x using both Agilent and Roche NimbleGen kits. Validation of a limited subset of single nucleotide variants (SNVs) previously revealed 98% accuracy for variants. For additional methods regarding variant processing, please see the Supplementary Methods.

Loss-Of-Function Variant Analyses

As performed in previous whole-exome studies of complex traits, we focused on loss-of-function single-nucleotide variants which are empirically defined as nonsense and essential splice-site mutations.^{26, 27} To exclude potentially indolent mutations from the analysis and call loss-of-function with high specificity, strong loss-of-functions mutations were prospectively defined as those LoF variants that affect all NCBI annotated isoforms for a given gene.²⁸ In addition, since nonsense mutations toward the 3' end may yield proteins with partial or full functionality, nonsense mutations were required to induce nonsense-mediated decay (occurring 50 base pairs before the last exon-exon junction).^{28, 29} For genes with only one coding exon, nonsense mutations had to truncate at least 50% of the protein. Variants of interest from study participants were validated through Sanger sequencing. Please see Supplementary Table 4 for PCR primers and conditions.

Somatic BRAF/KRAS Genotyping

Histology of SSAs were reviewed and confirmed (G.Y.L). SNaPshot genotyping assay was performed on DNA from one representative SSA from each enrolled patient, if not performed in the clinical setting. The assay consists of upfront multiplexed PCR followed by single-base extension reactions using the SNaPshot platform as previously reported.³⁰ The following bases were genotyped: *KRAS* c.34G, c.35G, c.37G, c.38G, c.181C, c.182A, c.183A, and *BRAF* c.1397G, c.1406G, c.1789C, c.1798G, c.1799T.

Protein-Protein Interaction Network

The 86 genes of the predefined set found mutated among the controls were used as inputs into the Broad Institute's Disease Association Protein-Protein Link Evaluator (<http://www.broadinstitute.org/mpg/dapple/dapple.php>).³¹ Fixed causal genes were listed as *ATM*, *ATR*, *CHEK1*, and *CHEK2*. Direct interactions were visualized and plotted.

Statistical Analysis

The cohort allelic sums test was employed to assess enrichment of strong LoF mutations of the gene set in cases compared to controls.³² A $P < 0.05$ was considered significant given that only one gene set was compared between cases and controls. To create an upperbound limit, each individual from the controls was assumed not to have more than one strong LoF mutation from the gene set. This assumption was necessary given that variant data provided for this cohort are genotype counts not localizable to any specific individuals. Any such variant in which the total number of controls assayed was short of 4300 individuals due to issues regarding coverage around a particular genomic region, the number of individuals affected was projected to 4300 based on the MAF. Among search of novel genes implicated by GWAS, a Bonferroni-corrected $P < 9.0 \times 10^{-5}$ was considered statistically significant given 555 individual gene comparisons made (Supplementary Table 2).

RNF43 Functional Studies

Creation of RNF43 stable knock-down pancreatic duct cells, DNA damage experiments, and western blot analyses are described in detail in the Supplementary Methods.

Results

Characteristics of Study Participants

We used a revised set of criteria based upon the World Health Organization criteria for serrated polyposis as a framework to define a robust genotype-phenotype relationship for sessile serrated adenomas (Table 1; see **Materials and Methods**).²¹ The clinical features of study participants are displayed in Table 2. Reaffirming the severe phenotypes selected, the median age at which study participants fulfilled enrollment criteria was considerably younger than the median age at which sporadic SSAs are found in the general population (52 vs. 61 years).³³ The number of SSAs ranged from 3 to 50, representative of the broad range of polyp multiplicity that fulfills current criteria for serrated polyposis (1, 5, or 20). Sixteen out of the twenty participants met WHO clinical criteria. Three individuals had personal histories of colon cancer, eleven demonstrated family histories of colon cancer, and eight had one or more extracolonic neoplasms prior to their satisfying enrollment criteria (Table 2 and Supplemental Figure 1). After histological review of all polyps, genotyping of one representative SSA from each individual was performed in 19/20 study participants given sample availability. Eighteen of nineteen genotyped SSAs carried the *BRAF V600E* mutation, whereas the SSA of Study Participant #18 did not harbor an identifiable mutation in *BRAF* or *KRAS*.

Loss-of-Function Mutations within Pathways of Oncogene-Induced Senescence

We used computational approaches to define a set of central regulators of OIS and compared the prevalence of strong LoF mutations between cases and controls (Materials and Methods). Consistent with prior genetic studies of serrated polyposis, no single gene was found to be mutated in multiple participants. However, we detected strong LoF mutations in five different genes (*ATM*, *TELO2*, *RBL1*, *XAF1*, and *PIF1*) within our predefined gene set in five individuals (Figure 1a). All variants were verified by Sanger sequencing (Supplemental Figure 2). Study participant #8, with a previously undescribed nonsense mutation in *ATM*, demonstrated a strong personal and family history of colorectal and extracolonic cancers (Figure 1b). While somatic nonsense mutations in *ATM* occur frequently in colon cancer, germline mutations have thus far been implicated in hereditary breast and pancreatic cancers.³⁴⁻³⁶ Study participant #5 possessed a 5' essential splice-site mutation in *TELO2* and a personal history of early-onset cholangiocarcinoma. *TELO2* encodes for the protein Tel2, which facilitates the stability and activation of ATM and ATR

through direct physical binding; loss of Tel2 expression inhibits Chk1/Chk2 activation in the setting of DNA damage.³⁷ Study participants #2 and #9 each possessing a first-degree relative with CRC, carried nonsense mutations in *RBL1* and *XAF1* (rs146752602), respectively (Figure 1b). Loss of *RBL1*, which encodes the p107 protein that is a key constituent of the p16-Rb pathway, facilitates the development of dysplasia in the intestinal epithelium, as well as Ras-mediated transformation.^{38, 39} *XAF1*, a binding partner and activator of Chk1, shows decreased expression in 40% of colon cancers, and loss of heterozygosity noted in 36%.^{40, 41} Decreased expression of *XAF1* has been also noted in esophageal and pancreatic cancers.^{42, 43} Study participant #13 harbored a nonsense mutation in *PIF1*, a DNA helicase which maintains genomic stability by facilitating Chk1 activation and negatively suppressing telomerase.^{44, 45}

Among controls, strong LoF mutations were found in 86/233 genes of the predefined set, and their network topology demonstrated extensive, direct protein-protein interactions (Figure 2a). Compatible with strong purifying selection, cumulative minor allele frequencies (MAF) of strong LoF mutations for each gene rarely exceeded 0.1%, and did not surpass 1.1% (Figure 2b and Supplementary Table 5). Overall, 25% of study participants harbored at least one strong LoF mutation in the gene set compared to 9.87% of controls (5/20 vs. 424/4300, odds ratio [OR]=3.0; 95% confidence interval [CI], 0.9–8.9; $P=.04$, Fisher's two-tailed exact test).

Loss-of-Function Analysis Facilitates Discovery of RNF43 as a High-Risk Variant for the Development of Multiple Serrated Polyps

From this significant enrichment, we next hypothesized that the remaining fifteen study participants possessed strong LoF mutations in genes not yet implicated in the regulation of OIS. Each study participant harbored approximately 5 rare, strong LoF mutations from the exome (Supplementary Table 6). To demonstrate the utility of LoF analysis for gene discovery, we searched for strong LoF mutations within genes containing loci implicated by genome-wide associated studies (GWAS) of all cancers. We searched among GWAS of all cancers instead of just colon cancer specific studies given that mechanisms of senescence are broadly applicable to all cancers. Of note, minimal overlap exists between our predefined gene set of OIS and the set of all genes with loci identified from GWAS of cancers, demonstrating that only a small fraction of genes containing GWAS loci (1.8%) have previously been linked to mechanisms of senescence (Supplementary Figure 3).

Two genes that contain GWAS loci were discovered with strong LoF mutations among the fifteen remaining study participants. Study participants #4 and #11 possessed the rare variant (rs199884004), *ULK4* R862X (2/20 vs. 45/4300; 10% vs. 1%; OR=10.5; 95% CI, 1.1–45.8, $P=0.02$). A missense variant in *ULK4* has already been associated with multiple myeloma.⁴⁶ While its function is unknown, expression of its paralog, *ULK3*, has been observed to be increased in Ras-induced senescence, and its overexpression also induces a senescence phenotype.⁴⁷ Study participants #6 and #12 harbored the identical, novel germline mutation, *RNF43* R113X (Figure 3a and 3b) and had family histories of colon cancer. This enrichment of strong LoF mutations in *RNF43* in our cohort reached statistical significance (2/20 vs. 1/4300; 10% vs. 0.02%; OR=460; 95% CI, 23.1 – 16384; $P=6.8 \times 10^{-5}$). *RNF43* has been implicated in a GWAS of pancreatic cancer among Japanese, and has been reported as among the most frequently mutated genes in cystic tumors of the pancreas.^{48, 49} More recently, knockout of *RNF43* has been demonstrated to contribute toward an intestinal polyposis phenotype in mice.⁵⁰

RNF43 expression has been shown to be increased in tubular adenomas and conventional adenocarcinomas, so we next examined the expression profiles of *RNF43* in SSAs and cancers arising from the serrated pathway to confirm a possible tumor suppressor role in this

setting. As a direct transcriptional target of Wnt signaling, RNF43 has been demonstrated to have a homeostatic role and tumor suppressive role by inducing endocytosis of Wnt receptors.⁵¹ Analyzing several published microarray datasets (see Supplemental Methods), we observed that in contrast to tubular adenomas, SSAs demonstrated no significant increase in RNF43 expression when compared to normal mucosa (Supplementary Figure 4). In addition, a direct comparison of RNF43 expression between SSAs and tubular adenomas demonstrated a statistically significant, 2.2-fold decrease in SSAs. Moreover, this ratio was sustained in independent microarray studies when comparing expression profiles of adenocarcinomas derived from the serrated pathway versus conventional adenocarcinomas. These findings demonstrate a relative reduction of RNF43 expression in the serrated pathway compared to the conventional pathway of colon carcinogenesis.

RNF43 is a Novel Regulator of the ATM-ATR-mediated DNA Damage Response Pathway

The strong statistical signal from our case-control analysis and prior observations linking RNF43 to UV-induced apoptosis prompted us to investigate the potential interplay between RNF43 and the DDR pathway in the context of precancerous lesions.⁵² We chose pancreatic duct cells expressing activated *Kras*^{G12D} from the endogenous promoter (generated from *Pdx1-Cre; LSL-KRAS*^{G12D} mice; Supplemental Methods) as a tractable *in vitro* model system. These cells have intact OIS checkpoint genes and provide a relevant experimental context to study the genetic interactions between *KRAS* and *RNF43* mutations, as these lesions are observed concurrently in a significant subset of pancreatic cystic tumors.

We generated matched lines with *RNF43* knockdown or control shRNA, and examined response to UV irradiation. *RNF43* depleted cells exhibited sharply diminished accumulation of γ -H2Ax and phosphorylated Chk1 compared to control cells within the first several hours of UV exposure (Figure 4c). This effect was also seen at variable doses of UV radiation (Supplementary Figure 5). *RNF43* knockdown and control cells exhibited comparable proliferation rates, indicating that the diminished levels of γ -H2Ax and phosphorylated Chk1 were not a consequence of cell cycle arrest from *RNF43* knockdown (Supplementary Figure 6). Thus, impairment of *RNF43* function in precancerous cells results in the dampening of the DDR pathway.

Discussion

In this study, we demonstrate that an excess of rare, strong LoF mutations (nonsense and splice-site) in genes that regulate primary mechanisms of OIS in the colon are associated with the development of multiple SSAs. To discover this association, we focused our analysis on rare variants. Recently, much of the genetic research in colon cancer has focused on the search for common variants. However, multiple GWAS and subsequent meta-analyses have not revealed genetic factors that can account for the overall heritability for colon cancer. Increasing evidence is emerging that a large fraction of this missing heritability for complex diseases may instead arise from a heterogeneous collection of rare variants.^{53, 54} Large scale exome sequencing projects have revealed that the bulk of protein-coding variation in populations is rare and has occurred recently, arising from rapid population growth outpacing selection pressures over the past 5,000 to 10,000 years.⁵⁵ Extreme phenotype design of whole-exome studies has facilitated the identification of rare variants in complex diseases.⁵⁶

To understand the genetic susceptibility to SSA development, we studied individuals who developed multiple SSAs, many of whom met criteria for serrated polyposis. We hypothesized that such individuals should be enriched with severe mutations. However, given the low prevalence of serrated polyposis syndrome in the general population, the genetic heterogeneity of the disorder, and the high false-positive rate of missense variant

prediction algorithms, obtaining sufficient statistical power for testing of individual rare or private variants is not feasible or practical.⁵⁷ To overcome this limitation, we leveraged observations regarding disease pathogenesis from prior animal and human studies and restricted our focus to disease-implicated pathways instead of individual genes to gain additional statistical power. We further restricted our analysis to empirically defined loss-of-function variants (nonsense and splice-site), which has been demonstrated to be a useful tool in exome-sequencing studies to implicate genes and pathways associated with complex traits.²⁷

This hypothesis-driven approach focusing on molecular pathways revealed that germline alterations in OIS pathways are associated with the formation of multiple SSAs. Of note, three of the initial five genes with strong LoF variants that were identified (*ATM*, *RBL1*, and *XAF1*) have been already established as genes implicated in colorectal carcinogenesis through human or animal studies. To reinforce the importance of these senescence mechanisms in disease pathogenesis, we subsequently demonstrated that remaining study participants harbored rare or novel LoF mutations in genes not yet implicated in these pathways. Through an integrative genomics approach, we identified *RNF43* as another high-risk gene for the development of multiple serrated polyps that may also regulate senescence through the DDR. Including *RNF43*, we identified strong LoF mutations in 35% of study participants.

These results have important implications for clinical practice. In contrast to established polyposis syndromes such as Familial Adenomatous Polyposis or *MUTYH*-Associated Polyposis, single gene tests are unlikely to capture the wide genetic heterogeneity associated with the development of multiple serrated polyps. Clinical whole-exome sequencing may be required to identify high-risk variants in patients. While identification of these rare variants in affected individuals may permit personalized approaches to identify at-risk relatives, counseling about the unique risks of each of these genes may be difficult given the numerous genes involved and their varying effect sizes. Also, any identified pathogenic mutation may not be described in large cohorts of controls or cases, preventing precise quantification of risk. As a result, as clinicians obtain more personalized genomic information, they may be required to infer recommendations from qualitative basic science and translational studies to guide their clinical management.

An additional challenge is that many of these rare variants may or already have significant pleiotropic effects on the risk of developing other extra-colonic cancers. In our cohort, a large fraction of study participants demonstrated strong personal or family histories of early-onset extra-colonic cancers (Supplementary Figure 1). Among the genes discovered in our cohort, *ATM* mutations have been already described in the development of hereditary breast and pancreatic cancers. *RNF43* is among the most frequently mutated genes in cystic neoplasms of the pancreas. While additional studies are required to formally quantify the risk of these pathways in the development of colon and extra-colonic cancers in family members, it is important to note that oncogene-induced senescence is relevant not only to SSAs but also to the development of most neoplasia. Thus far, pancreatic cancer has been linked to serrated polyposis in one cohort.¹² However, tissue specificity and penetrance of these cancers may be secondary to environmental exposures and epistatic relationships among other variants in the genome.

There are several limitations to our study. The first derives from the existing WHO criteria for serrated polyposis, which we used as a framework. These clinical criteria encompass a broad phenotype, incorporating different polyp multiplicities, polyp location, polyp size, and family history (Table 1). To maximize discovery of genetic contributors, we developed modified criteria. We omitted polyp size, as it is dependent on timing of endoscopy and

often an estimate by the endoscopist. In addition, serrated polyps are now more likely to be removed at sizes smaller than 1 cm due to their increased recognition. We used family history of CRC as a substitute for family history of serrated polyposis given the established risk among first-degree relatives and failure to recognize SSAs in older pathology reports. Another limitation of this study is that polyp or cancer histories of controls were not available and assumed to be negative. As a consequence, though, our calculated association of multiple SSAs with these LoF variants is likely an underestimate.

Our study is the first exome-wide genetic analysis of individuals who develop multiple SSAs, many of whom satisfy criteria for serrated polyposis syndrome. Utilizing a hypothesis-driven approach to exome sequencing, we demonstrate that such individuals harbor LoF mutations in established and novel regulators of OIS. In addition to laying the foundation for further refinement of genotype-phenotype relationships in SSA development, these findings provide a framework for studying the genetics of the broader set of malignancies occurring in these individuals and their families.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

DDR	DNA Damage Response
ESP6500	Exome Sequencing Project 6500 Cohort
GWAS	genome-wide associated studies
GATK	Genome Analysis Toolkit
HGNC	HUGO Gene Nomenclature Committee
MAF	minor allele frequency
MGH	Massachusetts General Hospital
NHLBI	National Heart, Lung, and Blood Institute
OIS	oncogene-induced senescence
SNV	single nucleotide variant
SNP	single nucleotide polymorphism
SSA	sessile serrated adenoma

Figure 1. Strong Loss-of-Function Mutations Among Study Participants

(A) Gene structure diagrams for all official isoforms of mutated genes. Vertical structures along the line demonstrate exons. Downward pointing arrows indicate the site of mutations. Isoforms are labeled with RefSeq IDs. (B) Pedigrees of study participants with identifiable mutations in the gene set and family histories of colon cancer. Ages are current as of time of publication. Study participants #5 and #13 did not have family histories of colon or extracolonic cancers and are not displayed.

Figure 2. Strong Loss-of-Function Mutations Among Controls

(A) Direct protein-protein interaction network of gene set mutations discovered in controls. Red halos designate genes also found mutated in cases. (B) Cumulative MAFs of each mutated gene in order of decreasing frequency. Arrows designate genes found in cases. The skewness was 8.8 and kurtosis was 92.1.

Figure 3. RNF43 Modulates the ATM-ATR-mediated DNA Damage Response Pathway
(A) Gene-structure diagram of the germline *RNF43* mutation identified from the study participants. Arrow demonstrates site of nonsense mutation. (B) Pedigrees of study participants with germline *RNF43* mutations. (C) Western blots of protein isolated from KRAS mutated pancreatic duct cells. Cells were stably transfected with short hairpin constructs targeting either GFP or RNF43, and exposed to UV irradiation as indicated. (D) Efficacy of RNF43 silencing in stably transfected pancreatic duct cells. Error bars designate standard error of the mean.

Table 1

Enrollment Criteria.

Revised World Health Organization Criteria for Serrated Polyposis		Enrollment Criteria	
1	At least five serrated polyps proximal to the sigmoid colon with two or more of these being >10 mm, or	1	At least five sessile serrated adenomas proximal to the sigmoid colon, or
2	Any number of serrated polyps proximal to the sigmoid colon in an individual who has a first-degree relative with serrated polyposis, or	2	Any number of sessile serrated adenomas proximal to the sigmoid colon in an individual with first degree relative with sessile serrated polyposis or colon cancer, or
3	>20 serrated polyps of any size, but distributed throughout the colon.	3	>20 sessile serrated adenomas, but distributed throughout the colon.

Fulfillment of any of the three criteria is sufficient for diagnosis or enrollment. Polyp counts are intended to be cumulative over time. Example of other types of serrated polyps include traditional serrated adenomas and microvesicular type hyperplastic polyps.

Table 2

Characteristics of the Study Participants

Participant Number	Age	Gender	SSAs	Personal History of CRC (age)	1° or 2° Relative with CRC (age)	Personal history of extracolonic malignancy (Age)	WHO Criteria (1,2,3)
1	57	Female	23	Yes (58)	No	Breast (45) Ovarian (55)	Yes (1&3)
2	45	Male	6	No	Father (56)	No	No
3	44	Male	5	No	No	No	Yes (1)
4	38	Female	5	No	Father (76)	No	Yes (1)
5	50	Male	7	No	No	Biliary (40)	Yes (1)
6	51	Female	>30	No	Father (70s)	No	Yes (1&3)
7	52	Male	9	No	No	No	Yes (1)
8	32	Female	7	Yes (32)	Uncles (40s)	Schwannoma (32)	Yes (1)
9	64	Female	10	No	Sister (51) Maternal Grandmother (60)	No	Yes (1)
10	52	Male	> 50*	Synchronous (52)	No	Germ Cell (41) Bladder (49)	Yes (1&3)
11	52	Male	10	No	No	No	Yes (1)
12	52	Female	7*	No	Maternal Uncle (70s) Maternal Aunt (70s)	No	Yes (1)
13	53	Male	9	No	No	No	Yes (1)
14	51	Male	8	No	Brother (58) Paternal Grandmother	Hodgkin's (34) Prostate (44)	Yes (1)
15	40	Female	3	No	Father (72)	No	No
16	57	Female	33	No	No	Hodgkin's	Yes (1,3)
17	66	Male	8	No	No	Melanoma (31)	Yes (1)
18	55	Female	6	No	Father (72)	No	No
19	49	Female	7	No	Maternal Uncle (45) Maternal Grandfather	No	No
20	56	Male	>25	No	Maternal Uncle (72)	No	Yes (1&3)

Age reported refers to the time at which the patient satisfied enrollment criteria. The polyp counts are current as of the time of submission.

* Designates individuals who underwent partial or total colectomy.