GASTROINTESTINAL, HEPATOBILIARY, AND PANCREATIC PATHOLOGY

Expression Profiling Reveals Involvement of WNT Pathway in the Malignant Progression of Sessile Serrated Adenomas

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Approximately 15% to 20% of colorectal cancers are developed through the serrated pathway of tumorigenesis, which is associated with *BRAF* mutation, CpG island methylation phenotype, and *MLH1* methylation. However, the detailed process of progression from sessile serrated adenoma (SSA) to dysplasia and carcinoma has not been elucidated. To further characterize mechanisms involved in the dysplastic progression of SSA, we investigated differential expressions of mRNAs between areas with and without dysplasia within the same SSA polyps. Significantly dysregulated genes in paired samples were applied for functional annotation and biological significance. The same lysates from a subset of matched samples were subjected for miRNA expression profiling. Differentially expressed miRNAs were determined, and their targeted mRNAs were compared in parallel to the list of differentially expressed mRNAs from an RNA sequencing study. Fourteen common mRNA targets were identified, which include *AXIN2*, a known indicator of WNT/ β -catenin pathway activation. Together, in this study, different genes, pathways, and biological processes involved in the initiation and progression of dysplasia in the serrated pathway are documented. One of the most significant findings is the involvement of the WNT/ β -catenin pathway in the dysplastic progression of SSAs with different genes being targeted in early versus advanced dysplasia. (*Am J Pathol* 2019, 189: 1732–1743; <https://doi.org/10.1016/j.ajpath.2019.05.009>)

Colorectal cancer (CRC) is the third most common malignant tumor and the fourth leading cause of cancer death worldwide.¹ For many years, the conventional adenoma to carcinoma sequence was known as the only route for colorectal carcinogenesis. However, emerging evidence indicates an alternative pathway of colorectal tumorigenesis, namely the serrated pathway, which accounts for approximately 15% to 20% of CRCs. Sessile serrated adenoma (SSA) represents a major precursor for the serrated pathway of CRC and accounts for almost 25% of all colorectal serrated lesions.^{2–4} Compared with the conventional pathway of colorectal tumorigenesis, the sequence of molecular events in the serrated pathway is less understood. Most early serrated lesions develop through *BRAF* (or to a lesser extent *KRAS*) mutations and CpG island methylation within the promoter region of multiple genes, a process that leads to CpG-island methylator phenotype (CIMP) and silencing of numerous tumor-related genes.^{5–7} CIMP can be

found in most SSAs and sporadic microsatellite instability-high (MSI-H) CRC cases, although it is not restricted to these groups.^{8–11} In a subset of the CIMP-positive serrated lesions, hypermethylation of CpG islands located in the promoter region of *MLH1* gene leads to mismatch repair deficiency and consequently microsatellite instability. Although the contribution of *BRAF* mutation, CIMP phenotype, and *MLH1* methylation in the development of sporadic MSI-H CRCs is well documented, the detailed process of progression from SSA to dysplasia and carcinoma remains elusive.

To investigate the mechanisms involved in the dysplastic progression of SSAs, the differential expressions of mRNAs

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and miRNAs in the regions of SSA and SSA with morphologic dysplasia were evaluated within the same polyp. The following challenges exist in assessing the expression profile of dysplastic SSAs: i) morphologic features are required for the diagnosis of dysplasia and therefore fresh tissue cannot be used; ii) areas of dysplasia in SSAs are typically small and yielded genomic material is often low; and iii) quality of RNA is suboptimal in formalin-fixed, paraffin-embedded (FFPE) tissue. A novel sensitive method for RNA sequencing, namely AmpliSeq transcriptome analysis, with optimized efficiency for detection of small amounts of RNA in FFPE tissue was used.^{12,13} For miRNA evaluation, a nanostring platform was used, which contained adequate probes to cover the most important target miRNAs (approximately 800 targets) and was previously reported to have superior efficiency in FFPE tissue.^{14,15} Significantly dysregulated genes were applied for functional annotation and biological significance using Ingenuity Pathway Analysis (IPA) software version 01-12¹⁴ (Qiagen, Hilden, Germany) and Database for Annotation, Visualization and Integrity Discovery (DAVID) version 6.8 (National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD; <https://david.ncifcrf.gov>). In a few prior reports, gene expression profiling of SSA was attempted.^{16–18} However, the focus of these studies was to identify genes involved in the development of SSA and not its dysplastic progression. This study is also unique with respect to side by side comparison of nondysplastic versus dysplastic components in each SSA polyp. Combined analysis of miRNA and mRNA data in parallel for the same individual polyps added more strength to the findings.

Materials and Methods

Tissue Selection, Inclusion Criteria, and Clinicopathologic Data

This study was approved by the Ethics Committee of the University of Calgary. A search was made in the University of Calgary database (from 2011 to 2015) for the colon polyps with the diagnosis of SSA with dysplasia, arising from the right side of the colon (cecum, ascending and transverse colon). The polyps were reviewed, and 21 SSA polyps in which dysplasia encompassed more than a single 10× field (>2 mm) were selected. These polyps include 12 SSA polyps with areas of low-grade dysplasia (SSA-LD group) and 9 SSA polyps that contained areas of high-grade dysplasia (SSA-HD group). In each group (SSA with or without dysplasia), tissue was harvested in the form of 1-mm cores. For the paired control group, tissues were collected from the nondysplastic SSA portion, adjacent but at least one 4× field (>5 mm) away from the dysplastic area of each polyp (SSA-ND group). This approach was performed to minimize the risk of cross-contamination.

Histologic criteria used for the diagnosis of SSA with dysplasia include i) the presence of SSA component within

the lesion, ii) an abrupt transition from SSA to SSA with unequivocal dysplasia, and iii) exclusion of cases with traditional serrated adenoma arising in an SSA. Low-grade conventional dysplasia is defined by narrow, elongated, hyperchromatic, pseudostratified nuclei with basophilic cytoplasm. High-grade dysplasia is defined by significant polymorphism, increased mitotic activity, and loss of polarity and architecture complexity, including cribriforming. Cases with serrated-type dysplasia are excluded. The clinicopathologic data, including age, sex, size, and location of the polyp in the colon, are summarized in [Supplemental Table S1](#). Briefly, SSA-LDs (12 polyps) were excised from six females (50%) and six males (50%), whereas SSA-HDs (nine polyps) were excised from five females (56%) and four males (44%). Of the SSA-LDs (12 polyps), eight (67%) were located in the ascending colon, three (25%) in the cecum, and one (8%) in transverse colon. In the SSA-HD group (nine polyps), six (67%) were in the ascending colon, two (22%) in the cecum, and one (11%) in the transverse colon. The mean size in SSA-LD group was 1.2 cm, whereas the mean size in the SSA-HD group was 1.3 cm. The mean age of patients in the SSA-LD group was 67.6 years, whereas the mean age was 70.6 years in SSA-HD group.

RNA Extraction, Integrity Assessment, mRNA Sequencing, and Data Analysis

RNA was extracted from formalin-fixed, paraffin-embedded (FFPE) tissue using an Ambion Kit for Nucleic Acid Isolation from FFPE (Thermo Fisher Scientific, Waltham, MA). In summary, duplicate 1-mm cores of tissue from the nondysplastic and dysplastic portions of each polyp were dissected from the FFPE blocks. Samples were deparaffinized with Xylene and washed with alcohol before RNA extraction. The quantity of extracted RNA samples was assessed using Nanodrop UV-VIS spectrophotometer (NanoDrop Technologies, Wilmington, DE), and the integrity of RNA was assessed using a Bio-analyzer 2100 and RNA Nano Chip assay (Agilent Technologies, Wilmington, DE). Whole transcriptome sequencing was outsourced to the next-generation sequencing laboratory in Dr. Djavad Mowafaghian Centre for Brain Health at the University of British Columbia and was performed using 100 ng of total RNA with AmpliSeq Transcriptome Human Gene Expression kit on an Ion Proton sequencer (Life Technologies, Carlsbad, CA).

For mRNA sequencing, the Ion Torrent Server version 5.0 (Thermo Fisher Scientific) was used to align the short reads in fastq files to the National Center for Biotechnology Information 37.1 reference genome. The AmpliSeq RNA plugin was used to generate absolute and normalized gene expression values. Genome annotation database and the quantification of the relative abundance of each transcript were reported as fragments per kilobases of transcript per million mapped reads. Qualified samples had a means $\pm$ SD of 10.4 ± 2.5 reads. Low-expression genes were filtered out as

suggested by Sha et al.¹⁹ Wilcoxon signed-rank test was used to compare matched samples (SSA versus SSA-LD and SSA versus SSA-HD). The *U* test was used to compare unpaired samples (SSA-LD versus SSA-HD). The Benjamini-Hochberg method was used to control the false discovery rate (FDR). The threshold of declaiming a gene to be differentially expressed [differentially expressed genes (DEG)] are based on $FDR \leq 0.05$ and $\log_2$ fold change (FC) absolute value > 1.5 . For cluster analysis, expression data were first scaled by SD for each gene. Euclidean distance was used for dissimilarity measure. Unsupervised, nonhierarchical clustering using the K-mean method was then applied.

For functional annotation and biological significance, the DEGs were analyzed with the IPA software. In addition, gene ontology analysis was also performed on the DEGs using the DAVID. $FDR \leq 0.05$ was used as the criteria for gene ontology category enrichment. RNA sequencing data have been deposited in the ArrayExpress database (European Molecular Biology Laboratory—European Bioinformatics Institute, Cambridge, UK; <https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-7960>, last accessed July 2, 2019).

NanoString Code Set Design and miRNA Expression Assay and Data Analysis

RNA samples extracted from four randomly selected SSAs with low-grade dysplasia and their associated SSAs without dysplasia were processed using an nCounter Human miRNA Expression Assay version 3 kit (NanoString Technologies, Seattle, WA) that contained probes and miTags for 822 important miRNAs related to the major pathways in neoplastic events. Briefly, probes were hybridized to 300 ng of total RNA extracts for 20 hours at 65°C and applied to the nCounter™ Prep Station for automated removal of excess probe and immobilization of probe-transcript complexes on a streptavidin-coated cartridge. Data were collected using the nCounter™ Digital Analyzer by counting the individual barcodes.

NanoString data processing was performed using nSolver Analysis Software version 3.0 (NanoString Technologies). Raw miRNA counts were normalized with ligation control. The background level of expression for each sample were calculated by the mean counts of negative control plus two SDs of the mean and were subtracted from the raw data counts of the sample. Normalized data were $\log_2$ -transformed and then used as input for further analysis.

The *U* test was used for analysis of statistically significant miRNAs between SSAs with low-grade dysplasia and their nondysplastic counterparts. The threshold of declaiming a miRNA to be differentially expressed are based on $P \leq 0.05$ and $\log_2$ FC absolute value > 1.5 .

SPSS software version 20.0 (IBM, Armonk, NY) was used for statistical evaluation, and Prism version 7.0 software (GraphPad Software Inc., La Jolla, CA) was used for graphing. nSolver software version 3.0 (NanoString Technologies) was used for the normalization of raw counts.

Nonhierarchical clustering, principle component analyses, and volcano plot were performed on R Package Bioconductor ComplexHeatmap Software version 3.7 (Bioconductor, Lund, Sweden), and Qlucore Omics Explorer version 3.2 (Qlucore, Lund, Sweden), and R Graphing Library (Plotly, Montreal, QC, Canada).

Quantitative PCR

Target and reference miRNA were reverse transcribed using the TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific) and the following specific TaqMan miRNA assays: has-miR-222-3p (002276), hsa-miR-191 (002299), and U6 snRNA (001973). Ten nanograms of total RNA was used as input for each reverse transcription reaction. miRNA expression was determined by quantitative PCR on an ABI 7500 Fast instrument (Thermo Fisher Scientific). Each singleplex assay was performed in duplicate, consisting of 1 μ L of 20 $\times$ TaqMan assay, 1.33 μ L of reverse transcription reaction, 10 μ L of TaqMan Universal PCR Master Mix (2 $\times$), and 7.67 μ L of nuclease-free water. The following cycling conditions were used: 50°C for a 2-minute hold, 95°C for a 10-minute hold, 95°C for 15 seconds, and 60°C for 1 minute for 40 cycles. miR-222-3p relative expression levels were calculated using the $\Delta\Delta C_t$ method and SDS software version 2.05 (Thermo Fisher Scientific). A comparison between the SSA and SSA-LD groups was performed using the *t*-test. Relative expression miR-191 and U6 served as internal reference controls for each assay.

Immunohistochemistry

Immunohistochemistry stains using anti- β -catenin antibody (dilution 1:1000, 30 $\times$ 30, high pH, clone 14; BD Bioscience, Franklin Lakes, NJ) were performed on 4- μ m sections of FFPE tissue using a Omnis platform (Dako Cytomation, Glostrup, Denmark). Slides were then counterstained and visualized by light microscopy. Slides were scored by two pathologists independently. Discrepant scores were resolved after joint evaluation of cases. β -catenin immunostain showed membranous, cytoplasmic, and nuclear staining patterns. The results of staining were considered positive for nuclear accumulation if $> 50\%$ of cells showed nuclear staining in longitudinally oriented crypts. Immunohistochemistry stains were performed on a separate independent cohort of 20 dysplastic SSAs comprising 10 SSA-LDs and 10 SSA-HDs. The original cohort could not be used as in many cases the dysplastic component was exhausted for RNA extraction. Nuclear β -catenin expression was compared between SSA-LD and SSA-HD using the Fisher exact test.

Methylation-Specific PCR

DNA was extracted from corresponding lysate material used for RNA extractions following the manufacturer's protocol

(QIAamp DNA Micro Kit, Qiagen). Five cases of randomly selected SSA-LD and five cases of SSA-HD were included for analysis of *AXIN2* promoter methylation. DNA concentration and purity were assessed by NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific). All samples had 260/280-nm absorbance values >1.9 . For each sample, 1 μ g of genomic DNA was bisulfite treated using the Epi-Test Fast Bisulfite Kit according to the manufacturer's protocol (Qiagen). Samples were eluted in 15 μ L of elution buffer, and 4 μ L served as template for each PCR reaction. The following primers pairs were used²⁰: *AXIN2* M forward 5'-ATATAGTTTACGCGTTGGGAGTGC-3' and *AXIN2* M reverse 5'-CACTCGACCAAAACGCACG-3' 112 bp; *AXIN2* UM forward 5'-ATAGTTTACGCGTTGGGAGTGT-3' and *AXIN2* UM reverse 5'-CCACTCAACCAAAACACACA-3' 113 bp. PCR reactions consisted of 5 μ L of 10 $\times$ HiFi PCR buffer, 2 μ L of 50 mmol/L $MgSO_4$, 1 μ L of 10 mmol/L dNTP mix, 1 μ L 10 μ mol/L forward primer, 1 μ L 10 μ mol/L reverse primer, and 0.2 μ L Platinum Taq DNA polymerase HiFi 5 U/ μ L (Thermo Fisher Scientific). Unmethylated PCR cycling conditions were as follows: 94°C 2 minutes initial denaturation followed by 94°C 15 seconds, 56°C annealing 30 seconds, and 68°C for 30 seconds for 35 cycles followed by 5 minutes of final extension at 68°C. The same conditions were used for the methylated reaction except annealing temperature was 62°C. PCR products were visualized on a Bioanalyzer 2100 instrument (Agilent, Santa Clara, CA) using a DNA 1000 assay kit according to manufacturer's protocol. Methylation of *AXIN2* promoter was compared between SSA-LD and SSA-HD groups using the Fisher exact test.

Results

Differentially Expressed Genes in SSA-LD and SSA-HD versus SSA-ND

To identify the genes that are dysregulated in dysplastic SSAs, 12 SSA-LD and 10 SSA-HD polyps were selected. One SSA-HD failed to show optimal reads and was therefore excluded from the study. The expression levels of mRNA between dysplastic areas of SSA polyps were compared with those in the nondysplastic (SSA-ND) portion of each SSA polyp. To reduce the large sets of data, two exclusion criteria were applied: $FDR \leq 0.05$ and $\log_2 FC$ absolute value >1.5 . From 20,812 genes that had abundant expression levels (fragments per kilobases of transcript per million mapped reads >1) by mRNA sequencing, 114 genes in the SSA-LD group and 153 genes in the SSA-HD group were differentially expressed compared with their nondysplastic counterparts (Supplemental Tables S2 and S3). In the SSA-LD group, these 114 DEGs included 54 up-regulated genes and 60 down-regulated genes (Figure 1A). In the SSA-HD group from 153 DEGs, 103 genes were up-regulated, and 50 genes were down-regulated (Figure 1B). Unsupervised, nonhierarchical clustering analysis of the 114

Figure 1 Volcano plot of 20,812 genes in sessile serrated adenoma—low grade (SSA-LD) (A) and sessile serrated adenoma—high grade (SSA-HD) (B) with mean fragments per kilobases of transcript per million mapped reads >1 and false discovery rate (FDR) ≤ 0.05 . Each circle corresponds to one gene. The x axis represents the gene $\log_2$ [SSA-LD or SSA-HD/SSA-ND (nondysplastic)] fold change (FC) value, and the y axis represents the $\log_{10}$ FDR value calculated from the Wilcoxon signed-rank test and Benjamini-Hochberg method between dysplastic portion of polyps and its nondysplastic counterpart. The pink circles represent the differentially expressed genes with the gene $\log_2$ value >1.5 or <-1.5 and the $\log_{10}$ FDR value ≤ 0.05 . The purple, green, and orange circles represent the genes with $\log_2 FC$ values between -1.5 and 1.5 or $\log_{10}$ FDR values >0.05 or both.

DEGs in the SSA-LD group and 153 DEGs in the SSA-HD group found that 12 of 12 SSA-LD samples and six of nine SSA-HD samples were clustered into distinct groups separated from their SSA counterparts in another group (Figure 2). These data demonstrate that SSA-LD and SSA-HD can generally, but not in every case, be distinguished from their adjacent nondysplastic components using large-scale gene expression analysis. Although the presence of occasional outliers in SSA-HD may represent alternative pathways involved in dysplasia in these polyps, the

Figure 2 Nonhierarchical clustering analysis of 114 differentially expressed genes (DEGs) in sessile serrated adenoma—low grade (SSA-LD) (A) and 153 DEGs in sessile serrated adenoma—high grade (SSA-HD) (B) compared with no dysplasia (SSA-ND).

possibility of nonpreferred quality of RNA in FFPE samples as a contributing factor cannot be excluded. Unpaired comparison was performed between the SSA-LD and SSA-HD groups. This comparison revealed three genes with significant differential expression, namely *LGALS1* (which express galectin-1), *Spock1*, and *TGFB2*.

Biological Processes Involved in Progression of SSA to SSA-LD

Analysis of the 114 differentially expressed genes in SSA with low-grade dysplasia found significant alterations in 15 key biological processes as defined by DAVID Bioinformatics and IPA. Nine of these biological processes were significantly up-regulated in SSA-LD, and six processes were down-regulated (Figure 3A). The up-regulated processes included cellular response to carbohydrate stimulus, positive regulation of glycolytic process, positive regulation of telomerase activity, regulation of calcium ion—dependent exocytosis, response to lipopolysaccharides, WNT signaling pathway, inflammatory response, immune response, and G-protein—coupled receptor signaling pathway. The down-regulated processes included negative regulation of cytokine production involved in inflammatory response, positive regulation of guanylate cyclase activity, cellular response to fluid shear stress, maintenance of gastrointestinal epithelium, brown fat cell differentiation, and regulation of intracellular pH. Enrichment of several canonical pathways were also highlighted by the pathway analysis (Supplemental Figure S1A), such as WNT/ β -catenin signaling ($P = 1.51 \times 10^{-3}$), granulocyte adhesion and diapedesis ($P = 1.8 \times 10^{-3}$), LXR/RXR activation ($P = 2.86 \times 10^{-3}$), agranulocyte adhesion and diapedesis ($P = 1.40 \times 10^{-2}$), and inhibition of matrix metalloproteases ($P = 1.53 \times 10^{-2}$). Some of the top significantly enriched cellular functions were cell-to-cell signaling and interaction ($P = 6.95 \times 10^{-5}$), cellular assembly and organization ($P = 6.95 \times 10^{-5}$), cellular development ($P = 1.39 \times 10^{-4}$), cellular growth and proliferation ($P = 1.39 \times 10^{-4}$), cell death and survival ($P = 4.8 \times 10^{-4}$), carbohydrate metabolism ($P = 8.24 \times 10^{-4}$), lipid metabolism ($P = 2.34 \times 10^{-3}$), nucleic acid metabolism ($P = 4.84 \times 10^{-3}$), amino acid metabolism ($P = 9.67 \times 10^{-3}$), and energy production ($P = 9.67 \times 10^{-3}$) (Supplemental Figure S1B).

Biological Processes Involved in Progression of SSA to SSA-HD

Analysis of the 153 differentially expressed genes in SSA-HD compared with their nondysplastic counterparts found significant alterations in 47 key biological processes as defined by DAVID Bioinformatics and IPA. Thirty-eight of these biological processes were significantly up-regulated in SSA-HD, and nine processes were down-regulated (Figure 3B).

Enrichment of several canonical pathways were also highlighted by the analysis (Supplemental Figure S2A), such as axonal guidance signaling ($P = 1.90 \times 10^{-3}$); role of osteoblasts, osteoclasts, and chondrocytes in rheumatoid arthritis ($P = 1.11 \times 10^{-3}$); WNT/ β -catenin signaling ($P = 1.17 \times 10^{-3}$); VDR/RXR activation ($P = 1.99 \times 10^{-2}$); and inhibition of matrix metalloproteases ($P = 2.34 \times 10^{-3}$). Some of the top significantly enriched cellular functions were cell cycle ($P = 1.60 \times 10^{-6}$); cellular assembly and organization ($P = 1.03 \times 10^{-5}$); DNA replication, recombination, and

A

Figure 3 Gene ontology enrichment analysis of biological processes of the differentially expressed genes between sessile serrated adenoma—low grade (A) or sessile serrated adenoma—high grade (B) and their nondysplastic counterparts. The biological processes were selected using false discovery rate ≤ 0.05 in Database, Annotation, Visualization and Integrated Discovery (DAVID) software version 6.8.

B

Figure 4 Relative expression levels of five differentially expressed genes (DEGs) involved in the WNT pathway in sessile serrated adenoma—low grade (SSA-LD) (A) and nonhierarchical clustering analysis of the same genes in SSA-LD (B) compared with no dysplasia (SSA-ND). Relative expression levels of six DEGs genes involved in the WNT pathway in sessile serrated adenoma—high grade (SSA-HD) (C) and nonhierarchical clustering analysis of the same genes in SSA-HD (D) compared with SSA-ND. * $P < 0.05$.

repair ($P = 1.03 \times 10^{-5}$); cell-to-cell signaling and interaction ($P = 6.51 \times 10^{-5}$); cellular growth and proliferation ($P = 6.51 \times 10^{-5}$); cell death and survival ($P = 1.38 \times 10^{-4}$); lipid metabolism ($P = 1.38 \times 10^{-3}$); protein synthesis ($P = 3.24 \times 10^{-4}$); carbohydrate metabolism ($P = 7.67 \times 10^{-4}$); amino acid metabolism ($P = 8.03 \times 10^{-4}$); and nucleic acid metabolism ($P = 9.46 \times 10^{-4}$) (Supplemental Figure S2B).

Activation of WNT/ β -Catenin Pathway in SSA-LD and SSA-HD Groups

Analysis of the DEG by IPA software highlighted five genes involved in WNT pathway, which were overexpressed in the SSA-LD group compared with their nondysplastic counterparts. These genes were *MYC*, *MMP7*, *AXIN2*, *UBD*, and *SOX14* (Figure 4A). *MYC*, *MMP7*, and *AXIN2* are known downstream gene targets of the WNT pathway.²¹ *UBD* is involved in the destruction process of β -catenin,²¹ and the mechanism of WNT pathway regulation by *SOX14* is unknown. Unsupervised, nonhierarchical analysis of these five DEGs found that 11 of 12 SSA-LD samples were clustered into a distinct group separate from their SSA-ND counterparts (Figure 4B), which demonstrates that SSA-LD can generally be distinguished from their adjacent nondysplastic sample using expression analysis of these five genes.

Although the data indicate significant up-regulation of downstream effector genes involved in the WNT pathway, there is no evidence of involvement of more upstream WNT signaling genes (eg, *WNT*, *FZD*, *LRP5/6* families of receptor, and *DVL*) in progression of SSA-ND to SSA-LD (Supplemental Figure S3, A–C). In addition, the expression levels of WNT pathway–mediated T-cell factor/lymphoid enhancer-binding factor gene family are not affected (Supplemental Figure S3D). Among genes

involved in the β -catenin destruction complex/pathway, two genes (*AXIN2* and *UBD*) were up-regulated in SSA-LD compared with SSA-ND. No significant changes were identified in expression levels of *AXIN1*, *APC*, *GSK-3*, *CSNK1*, *PP2A*, and *BTRC* (Supplemental Figure S4).

In SSA-HD group, six genes involved in the WNT pathway were up-regulated compared with their SSA-ND matched samples, including the two downstream genes *MYC* and *MMP7*, a WNT pathway ligand *WNT2*, *UBD*, *SOX11*, and *TGFB3* (Figure 4C). Unsupervised, nonhierarchical analysis of these six DEGs in the SSA-HD group found that seven of nine SSA-HD samples were clustered into a distinct group separated from their SSA-ND counterparts (Figure 4D). This finding indicates that most SSA-HDs can be distinguished from their adjacent nondysplastic SSA by expression analysis of these six genes. These results confirm that the WNT pathway remains activated in SSA-HD. In addition, up-regulation of *WNT2*, a ligand known to drive the β -catenin signaling pathway, is particularly interesting because this phenomenon can explain the manner of WNT pathway activation in SSA-HD group.

To examine WNT pathway activation at the protein level, immunohistochemistry was used to evaluate nuclear β -catenin expression in a cohort of 20 dysplastic SSAs, including 10 SSA-LDs and 10 SSA-HDs. Positive nuclear β -catenin was found in 6 of 10 SSA-LDs (60%) and 7 of 10 SSA-HDs (70%). Background nondysplastic SSAs had only 10% ($n = 2/20$) nuclear staining for β -catenin (Supplemental Figure S5). Immunohistochemistry revealed significantly higher nuclear β -catenin expression in SSA-LD ($P = 0.003$) and SSA-HD ($P = 0.0007$) compared with nondysplastic SSA. No difference in nuclear β -catenin expression was found between SSA-LD and SSA-HD ($P = 0.63$).

The analysis found up-regulation of *AXIN2* in SSA-LDs but not SSA-HDs. Using methylation-specific PCR (MS-PCR),

Figure 5 Relative expression levels of six miRNA down-regulated in sessile serrated adenoma—low grade (SSA-LD). Four SSA-LD polyps were selected, and the expression levels of 822 miRNA were evaluated and compared in dysplastic versus nondysplastic (SSA-ND) counterparts. * $P < 0.05$.

methylation of *AXIN2* was compared between SSA-LD and SSA-HD as a possible mechanism of *AXIN2* regulation in this setting. Five randomly selected cases from each of SSA-LD and SSA-HD groups were included for MS-PCR. One case from each group failed amplification. Methylation of *AXIN2* was detected in four of four SSA-LDs and three of four SSA-HDs ($P = 0.28$) (Supplemental Figure S6).

MicroRNA Expression Profile Highlights Down-Regulation of miR-222-3p in the SSA-LD Group with Specific Target mRNA *AXIN2* in the WNT Pathway

To identify dysregulated miRNA and their mRNA targets in dysplastic SSAs, five SSA-LD polyps were randomly

selected and miRNA expression levels in the dysplastic portion of the polyps were compared with those in the matched nondysplastic portion of each polyp, using NanoString platform. One sample failed to show optimal reads and was therefore excluded from the study. Two exclusion criteria were applied to reduce the large set of data: FDR ≤ 0.05 and $\log_2$ FC absolute value > 1.5 . From 822 miRNAs, which showed abundant expression levels, six miRNAs in the SSA-LD group were down-regulated compared with their nondysplastic counterparts (Figure 5). These miRNAs include miR-222-3p, miR-942-3p, miR-378g, miR-378i, miR-548j-5p, and miR-99a-5p. Many miRNAs were up-regulated in the SSA-LD group compared with the nondysplastic component, but the differential expressions were found to be insignificant after statistical analysis.

Next, the mRNA targets of these six down-regulated miRNAs were analyzed using IPA software. The IPA mRNA target library highlighted 323 target mRNAs involved in cancer pathogenesis for these six miRNAs. However, when the list of differentially expressed mRNA in the SSA-LD group was paired with these six down-regulated miRNAs, only 14 mRNAs were matched. The list of these 14 mRNA is given in Table 1. From these 14 targeted mRNAs, only one, *AXIN2*, is known to be involved in the WNT/ β -catenin pathway, which is targeted by miR-222-3p. To validate these findings, the expression level of miR-222-3p were examined in the rest of the SSA-LD cases that were not subjected to NanoString analysis. Quantitative RT-PCR studies found significantly decreased miR-222-3p in SSA-LDs compared with their nondysplastic counterparts ($P = 0.033$) (Supplemental Figure S7).

Discussion

In this study, a high-throughput and sensitive mRNA sequencing technology was used to compare the whole

Table 1 List of Five Down-Regulated miRNA with Their Paired Up-Regulated mRNA Targets in SSA Polyps with Areas of Low-Grade Dysplasia Group

Down-regulated miRNA	Adjusted P value	$\log_2$ FC	Targeted mRNA	Adjusted P value	$\log_2$ FC
miR-222-3p	0.0188	-2.325	<i>AQP3</i>	0.044	2.145
miR-222-3p	0.0188	-2.325	<i>AXIN2</i>	0.015	2.148
miR-222-3p	0.0188	-2.325	<i>RAB15</i>	0.015	1.594
miR-222-3p	0.0188	-2.325	<i>SYBU</i>	0.015	1.550
miR-942-3p	0.0260	-1.72	<i>DSG3</i>	0.044	1.742
miR-942-3p	0.0260	-1.72	<i>KCNH8</i>	0.015	1.676
miR-942-3p	0.0260	-1.72	<i>RNF183</i>	0.017	1.981
miR-378g	0.0286	-1.523	<i>APLN</i>	0.017	1.604
miR-378g	0.0286	-1.523	<i>CBX2</i>	0.025	2.359
miR-378g	0.0286	-1.523	<i>ETV4</i>	0.015	1.677
miR-378g	0.0286	-1.523	<i>S100A2</i>	0.017	1.641
miR-548j-5p	0.0299	-2.149	<i>AQP3</i>	0.044	2.145
miR-548j-5p	0.0299	-2.149	<i>CXCL5</i>	0.044	3.245
miR-99a-5P	0.0286	-1.709	<i>SRCIN1</i>	0.044	1.821

The threshold set for a both miRNA and mRNA to be declared differentially expressed were false discovery rate ≤ 0.05 and $\log_2$ FC absolute value > 1.5 . FC, fold change.

transcriptome in dysplastic versus nondysplastic regions of each SSA polyp. To strengthen the study findings, the miRNA expression profile was also examined in parallel for a subset of polyps, and their mRNA targets were correlated with mRNA sequencing findings. This work aims to identify genes, biological processes, and signaling pathways that are involved in the progression of SSA-LD to SSA-HD. Two specific approaches were used to minimize the background genomic noise. First, by selecting SSA polyps only from the right side of the colon, the heterogeneity of SSA polyps known to be present between left- and right-sided SSAs was excluded. Second, by comparing dysplasia (low or high grade) to the adjacent nondysplastic area within the same polyp, the background genetic differences that might be present in polyps with similar stages of progression in different individuals or within one patient were minimized.

Numerous biological processes involved in rapid energy retrieval that were enriched in both the SSA-LD and SSA-HD groups were identified. These processes include enhancement of cellular response to carbohydrate stimulus, glycolytic activity, regulation of cAMP metabolic processes, cAMP-mediated signaling, and energy reserve metabolic processes. In contrast, biological processes that are involved in more time-consuming energy retrieval or energy-consuming activities, such as oxidation-reduction reaction, lipoprotein transport, metabolism, biosynthetic processes, androgen and steroid metabolic processes, and brown fat cell differentiation, were reduced. These metabolic changes are all part of the well-known Warburg effect, which provides an immediate source of energy for rapidly growing neoplastic cells. In addition, multiple other biological processes were also enriched in both dysplastic groups, which are involved in enhancement of cell proliferation, including DNA replication, G1/S transition of mitotic cell cycle, chromosomal organization, chromosomal segregation, and mitotic nuclear division. Furthermore, in the SSA-LD group, positive regulation of telomerase activity was enriched, which is consistent with cell senescence, one of the well-known features of neoplasia. Apart from these changes, and specifically in the SSA-HD group, some other biological processes were enriched, which are involved in the enhancement of the ability of the neoplastic cells to invade. These processes include spindle cell organization, extracellular matrix disassembly, collagen catabolic process, collagen fibril organogenesis, and negative regulation of cell adhesion. All the above changes are a fundamental alteration in cell physiology, which is considered the hallmark of cancer.

Activation of WNT/ β -catenin pathways has an established role in conventional adenoma pathway mainly through *APC* and *CTNNB1* mutations. Recently, several studies have reported that activation of the WNT/ β -catenin pathway is involved in the development of dysplasia in SSA.^{22–24} Other studies failed to detect evidence of WNT/ β -catenin pathway activation in SSAs or sporadic MSI-H

CRCs arising from SSAs.^{25,26} The mechanism of WNT/ β -catenin activation in the serrated pathway has not been clearly elucidated. In contrast to the conventional adenoma pathway, mutations in the *APC* and *CTNNB1* genes are rare in the serrated pathway.^{22,24,27} Mutational analysis of the *APC* gene was not attempted in this study for two reasons: i) paucity of genomic material derived from targeted tissue and ii) mutational landscape of the *APC* gene in the serrated pathway has been extensively studied in the literature. More recent reports provided evidence of the presence of other mutations that affect the WNT pathway in the serrated pathway, including *RNF43* mutation. *RNF43* is a ubiquitin E3 ligase that down-regulates the WNT receptors, and therefore its inactivation up-regulates this pathway.²⁸ Recently, it has been reported that a minor subset of SSAs have *RNF43* mutations^{29,30} and the *RNF43* mutation was more frequently detected in SSAs with dysplasia compared with SSA.²⁴ Our results support the activation of the WNT/ β -catenin signaling pathway in low- and high-grade dysplasia evidenced by overexpression of the downstream B-catenin–targeted genes, including *C-MYC*, *MMP7*, and *AXIN2*. WNT/ β -catenin activation is an early event because it was detected in low-grade dysplasia and likely is required for maintaining neoplastic potential because it continues to be present in the high-grade dysplasia stage. Immunohistochemistry using β -catenin antibody in a small cohort of dysplastic SSAs confirmed WNT/ β -catenin activation in both SSA-LDs and SSA-HDs. No changes were observed in the expression levels of *APC*, *CTNNB1*, or *RNF43*. However, this study cannot rule out the possibility of *RNF43* involvement in the progression of SSA through loss of function mutations (without expression loss). Interestingly, this study highlighted another potential mechanism for activation of WNT/ β -catenin pathway in SSA-HD through up-regulation of *WNT2*. *WNT2* is a member of the WNT family previously reported to activate the canonical WNT/ β -catenin pathway in the conventional CRC pathway. The mechanism of *WNT2* up-regulation is not clear.³¹ However, recent studies suggest a role for stromal fibroblasts in *WNT2* up-regulation in conventional adenoma-carcinoma sequence.³² Therefore, it is plausible that stromal fibroblasts in the setting of dysplastic SSA activate the WNT/ β -catenin pathway in neoplastic epithelial cells through paracrine secretion of *WNT2*.

A significant positive trend in the expression of metalloproteinase *MMP7* from SSA-LD to SSA-HD was noted in our experiment. An overexpression of *MMP7* has been reported as a prognostic marker, and its increased expression or serum level is linked to metastatic disease.^{33–36} These results are in line with those reported by Polistena et al³⁷ in which a positive trend in the expression of *MMP7* was found in the conventional adenoma-carcinoma pathway. This finding is believed to be one of the alterations in the neoplastic process to acquire the ability of invasion. The role of *MMP7* in serrated CRC has not been investigated in detail; however, its overexpression has been reported in

MSI-H CRC through WNT/ β -catenin signaling as a marker of activation of this pathway.³⁸

Unpaired comparison between the SSA-LD and SSA-HD groups revealed three genes with significant differential expression, namely *LGALS1* (which express galectin-1), *Spock1*, and *TGFB2*. *LGALS1* is involved in cell adhesions, interaction with extracellular matrix, migration, angiogenesis, and metastasis. The role of *LGALS1* in progression and metastasis of CRC is documented in the literature.^{39–41} *Spock1* is involved in proliferation, adhesion, and cell-matrix interaction. Overexpression of *Spock1* in CRC is also documented in the literature.^{42,43}

There is now emerging evidence to suggest that alterations in the expression of small noncoding RNA genes, including miRNA, can also lead to carcinogenesis. The role of microRNA-mRNA interactions in CRC progression has been described in great detail elsewhere.⁴⁴ To our knowledge, no prior study compared the miRNA expression profile between SSA without dysplasia and SSA with dysplasia. miRNA evaluation was performed on the same lysates from paired samples of SSA and morphologic dysplasia that were sent for mRNA sequencing. With the use of stringent criteria, differentially expressed miRNAs were determined and their targeted mRNAs were compared with the data from mRNA expression profiling. Fourteen mRNAs were found to be common targets in both miRNA and mRNA data. The involvement of most of these genes, including *AQP3*, *RAB15*, *DSG3*, *KCNH8*, *RNF183*, *APLN*, *CBX2*, *S100A2*, *CXCL5*, and *SRCIN 1* in development and/or progression of CRC, has been documented in the literature.^{45–55} Among these targets, the association of miR-222-3p and enhanced expression of its target *AXIN 2* is specifically interesting. AXINs are the scaffolding proteins that are required for the formation of β -catenin destruction complex. Their function reduces the β -catenin bioavailability and consequently negatively affects the WNT/ β -catenin signaling pathway.⁵⁶ Therefore, their overexpression argues against the WNT pathway activity. However, *AXIN2* is a well-characterized target of WNT signaling and is expressed in a WNT/ β -catenin signaling-dependent manner as part of a negative feedback loop to control the WNT/ β -catenin signaling.⁵⁷ Therefore, up-regulation of *AXIN2* indeed is a hallmark of WNT/ β -catenin pathway activation. The overexpression of *AXIN2* has been previously reported in various tumors, including CRC.⁵⁸ Genome-scale exome sequencing analysis conducted by the Cancer Genome Atlas Project has reported that in MSI-H colon cancers *AXIN2* has altered through WNT/ β -catenin signaling.³⁸ However, in this study, despite *AXIN2* up-regulation in SSA-LD, this alteration was absent in the SSA-HD group. It has recently been reported that *AXIN2* is silenced in MSI-H CRC. It has been argued that the methylation as part of the CIMP phenotype can extend into the promoter region of *AXIN2*, leading to the gradual suppression of the *AXIN2* gene expression, which contributes to a step-wise acquisition of the epigenetic features seen in sporadic MSI-H colon cancer.²⁰ In addition, Murakami et al²³ found a

stepwise increment of *AXIN2* methylation with progression to high-grade dysplasia and submucosal carcinoma, resulting in silencing of *AXIN2* in MSI-H CRC. *AXIN2* methylation was compared by MS-PCR using lysates of five SSA-LDs and five SSA-HDs. There was no significant difference in the number of cases with methylated *AXIN2* between the two groups. However, MS-PCR is a semiquantitative method for analysis of methylation; therefore, the difference in methylation as a cause of *AXIN2* expression cannot be completely excluded.

In this study, the whole genome transcriptome as well as miRNAs in SSA with dysplasia were compared with their nondysplastic counterparts. The unique approach, discussed above, has reduced the background noise from genetic variations. Different genes, pathways, and biological processes involved in the initiation and progression of dysplasia were documented. The results confirmed that the WNT/ β -catenin pathway is activated in the early stage of dysplasia (ie, low-grade dysplasia) in the serrated pathway of tumorigenesis. *WNT2* and *AXIN2* were identified as markers of the WNT/ β -catenin pathway activation in the serrated pathway, and for the first time, five miRNA and their mRNA targets that are differentially expressed in SSA-LD compared with their nondysplastic counterparts have been introduced.

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Supplemental Data

Supplemental material for this article can be found at <http://doi.org/10.1016/j.ajpath.2019.05.009>.

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