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BOSTON UNIVERSITY

SCHOOL OF MEDICINE

Thesis

EXPRESSION OF COHESIN PROTEINS AND NANO-ARCHITECTURAL CHANGES IN RECTAL MUCOSA TO ASSESS RISK OF COLON CANCER BASED ON FIELD CARCINOGENESIS

by

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B.S., Tulane University, 2008

Submitted in partial fulfillment of the

requirements for the degree of

Master of Science

2014

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DEDICATION

I would like to dedicate this work to my Papa Bob—

For teaching me to always know more today than I did yesterday.

ACKNOWLEDGMENTS

Without certain people, I would not have had the opportunity to write this thesis. Thank you, Dr. Park, for being a constant source of inspiration and encouragement, always pushing me to reach my full potential. To Dr. Roy, one of the most brilliant minds I have encountered in the field of medicine—Thank you for your constant support, guidance, and continuous optimism. It has been a privilege to work with you. To Mart Dela Cruz, without whom this thesis would not exist, you went above and beyond in helping make this thesis a reality. I would like to also mention Ramesh Wali, Navneet Momi, Ramya Kuchibhatla, Jaclyn Weinstein, Lisa Jepeal, and Beth Parker for all your help throughout this process. I would like to especially thank Dr. Calderwood and the rest of the endoscopic clinicians for exposing me to a new field of medicine and your active support in my research. To all the nurses and technicians in the endoscopy unit—I cannot thank you enough for all your help and hard work. To my אמא, for always believing in me even when I did not.

EXPRESSION OF COHESIN PROTEINS AND NANO-ARCHITECTURAL CHANGES IN RECTAL MUCOSA TO ASSESS RISK OF COLON CANCER BASED ON FIELD CARCINOGENESIS

ARI B. DAVIS

ABSTRACT

With 50,310 related deaths this year, colorectal cancer (CRC) has emerged as the second largest cause of cancer related deaths among Americans. While 70 million Americans are considered at-risk of developing CRC, it is highly curable if detected early. Cohesin proteins, which hold sister chromatids together during replication, have emerged as a potential biomarker in multiple cancer lines. Because of their probable role in DNA replication, DNA repair, chromatin nanoarchitecture, and gene expression, this paper assessed whether cohesion proteins could be used as a potential biomarker for colorectal cancer risk stratification. While cohesin protein mutations have been reported in different cancers and involved in chromosomal instability, its role in early cancer formation has yet to be observed. Using immunohistochemical and Quantitative Real Time PCR analysis, this thesis assessed the protein and RNA expression levels of cohesin proteins SA-1, NIPBL, and SMC3 from human biopsies at different stages and locations of colorectal cancer development. The results showed that SA-1, a structural cohesion subunit, was significantly ($p<0.01$) down regulated in cancerous compared to normal tissue. The SA-1 protein was also down regulated in the involved mucosa adjacent to

CRC polyps. The cohesion loading protein, NIPBL, was also significantly ($p<0.01$) under expressed in cancerous versus normal tissue. The RNA expression analysis of rectal mucosa showed that SMC3 and SA-1 was over expressed two fold in patients harboring hyperplastic and adenomous polyps, giving evidence that cohesin proteins are differentially expressed throughout the field of carcinogenesis. Our results demonstrate for the first time that cohesion dysregulation is an early event in human colorectal cancer development and may serve as an important biomarker of field carcinogenesis.

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LIST OF ABBREVIATIONS

ACF .... Adenomatosis polyposis coli

BCP Bromo-3-cholopropane

BMC .... Boston Medical Center

cDNA .... Complementary DNA

CIN .... Chromosomal instability

CdLS .... Cornelia de Lange syndrome

CRC .... Colorectal cancer

CTCF .... CCCTC binding factor

EGF .... Epithelial growth factor

IHC .... Immunohistochemistry

PBS .... Phosphate buffered saline

PCR .... Polymerase chain reaction

SMC .... Structural maintenance proteins

I. INTRODUCTION

1.1 Background

With approximately 50,310 deaths in 2014, colorectal cancer (CRC) has emerged as the second leading cause of cancer-related deaths in the United States and worldwide (Siegel et al., 2014.). The disease prognosis is highly dependent on the stage at which the disease is detected. While patients with localized CRC have a 5-year survival rate of up to 90%, the 5-year survival rate for patients with metastasized CRC can be as low as 8% (Coppedè, 2014). As well, while 25% of patients with CRC have a family history of the disease, the majority of cases (75%) are sporadic (Hudler et al., 2014). Because of these factors, it is widely accepted that the best approach to reducing the mortality rate is through a reduction of advanced disease by screening the average risk population. Modern screening procedures achieve this goal by the detection and removal of early-stage neoplastic polyps. This ideology comes from the concept of the adenoma-carcinoma sequence, a term that describes the step-wise progression of normal mucosa to carcinoma over a period where the tissue accumulates genetic and epigenetic alterations (Carey et al., 2002).

Colorectal cancer screening options in the average-risk population fall into two main categories: stool tests that detect occult blood or exfoliated DNA and structural exams, which include flexible sigmoidoscopy, colonoscopy, and CT colography (virtual colonoscopy) exams. Fecal occult blood tests (FOBTs) have begun to decline in clinical use, partly due to it low sensitivity for advanced neoplastic polyps, which are defined as advanced adenomas and carcinomas (Roy et al., 2006). The virtual colonoscopy, while

showing promising results, is still undergoing validation studies and is unable to excise a polyp if found. The current gold standard for CRC screening is a colonoscopy procedure which directly inspects the mucosal lining of the entire colon and allows the clinician to evaluate and remove precancerous polyps. The American Cancer Society's "Guidelines for the Early Detection of Cancer" recommends that beginning at the age of 50, men and women should begin structural colon examination. While this screening procedure is extremely effective in detecting advanced neoplastic lesions and reducing the incidence of CRC by 65%, only a quarter of the eligible screening population undergo a colonoscopy screening procedure (Roy et al., 2009). Of patients that have colonoscopies, only 20-30% have adenomas. In addition, the procedure is invasive and requires one or more days of dietary preparation and bowel cleansing with poor bowel prep leading to variable performance of the test. These factors can inhibit the ability of the procedure to detect polyps. A recent study (Singh et al., 2010) found that nearly 1 in 13 CRCs were missed on initial colonoscopic examination. With over 100 million Americans at risk of developing CRC along with the time, economic, and discomfort set backs of the current screening mechanism, it remains clear that an alternative prescreening approach needs to be implemented.

1.2 Field carcinogenesis

Field carcinogenesis (also known as field effect or field cancerization) is a phenomenon that has many clinical implications for risk stratification. Field carcinogenesis is the idea that sequential genetic and environmental risk factors transform

an entire organ into a fertile zone for cells to undergo neoplasia and eventually tumor formation (Mutyal et al., 2013). While some cells in this mutational field become diagnosed histopathologically as a malignancy, it has been shown that other cells throughout the organ exposed to the same epigenetic and genetic factors display many of the same mutations as the cancerous cells (Backman et al, 2013). Therefore, these genetically transformed but histologically normal cells can be used to assess the risk of cancer formation. From a clinical perspective, field carcinogenesis has two main advantages in risk stratification. Since field carcinogenesis is not localized to a specific part of the colon, the markers for field carcinogenesis can be seen throughout the mucosa of the colon. Therefore, the rectal mucosa can be used as a surrogate site without the use of an endoscope or bowel purge. This would make screening less time intensive, more bearable for the patient, and less expensive. Secondly, since the genetic markers seen in field carcinogenesis throughout the mucosa of an organ are the precursors to neoplasia and tumor development, field carcinogenesis directly correlates to the risk of developing CRC. As opposed to attempting to preform colonoscopic procedures on the entire at risk population, which is remarkably inefficient with 6% of patients having advanced adenomas (Roy et al., 2009), using the field effect to pre-screen for patients at higher risk of developing CRC would highly increase the efficiency of screening and decrease the mortality of the disease.

1.3 Colorectal Biomarkers

Colorectal cancer develops slowly through a complex series of changes in cellular proliferation and differentiation that transforms normal flat mucosa into adenocarcinomas, making the colon highly susceptible to the field effect (Fig. 1). The transformational stages are normal flat mucosa, aberrant crypt foci, adenoma with low-grade dysplasia, adenoma with high-grade dysplasia, and adenocarcinoma (Bernstein et al

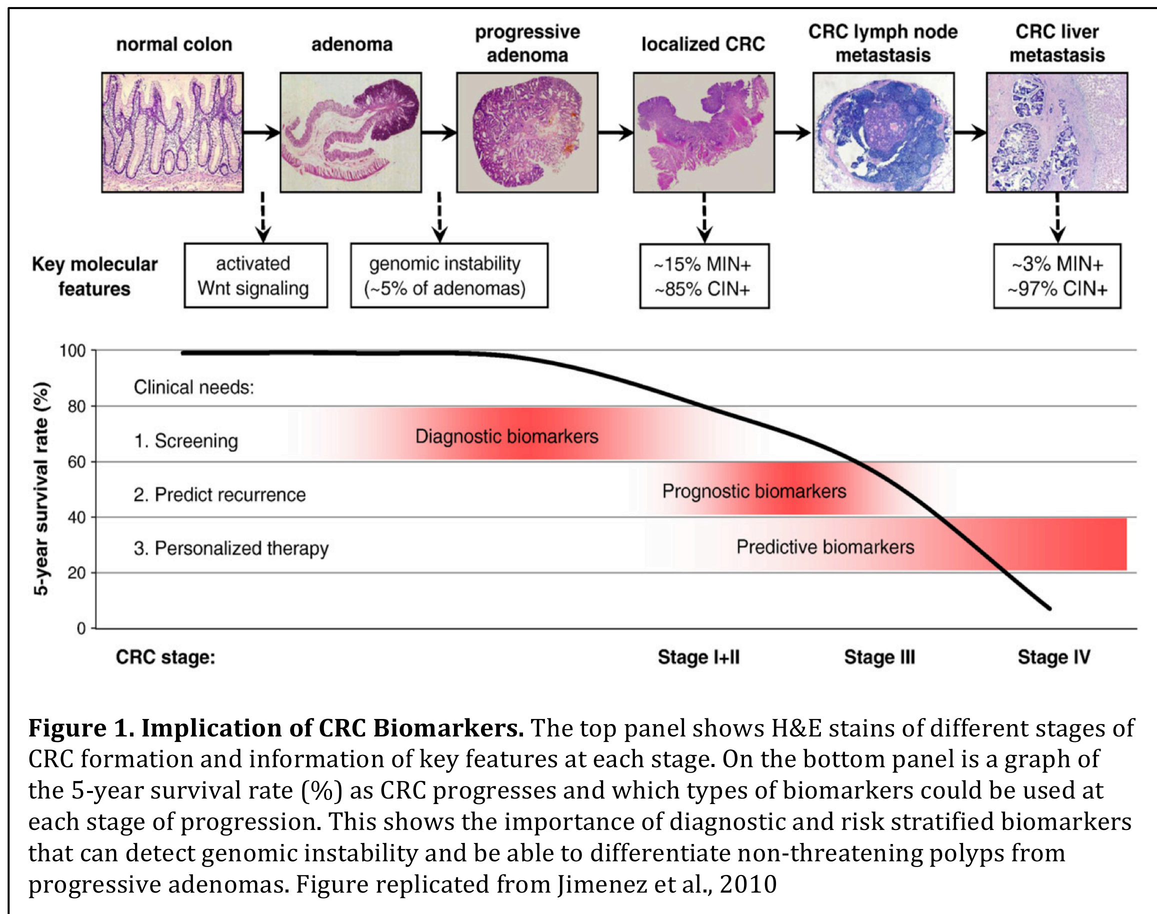

Figure 1. Implication of CRC Biomarkers. The top panel shows H&E stains of different stages of CRC formation and information of key features at each stage. On the bottom panel is a graph of the 5-year survival rate (%) as CRC progresses and which types of biomarkers could be used at each stage of progression. This shows the importance of diagnostic and risk stratified biomarkers that can detect genomic instability and be able to differentiate non-threatening polyps from progressive adenomas. Figure replicated from Jimenez et al., 2010

2008). The cellular changes in histologically normal mucosa manifest themselves as micro and nan-architectural changes that effect gene expression and cellular processes and can be used as clinical biomarkers (Dakubo et al., 2007). These biomarkers can be

classified into multiple distinct groups and their presence in distal normal appearing mucosa is predictive of adenomas elsewhere in the colon.

- **Morphological**: The presence of rectal aberrant crypt foci (ACF)
- **Micro-architectural:** Karyometrically identified nuclear parameters
- **Biochemically:** Activity of protein kinase C (PKC), ornithine decarboxylase, and mucus disaccharide
- **Immunohistochemically:** Loss of cytochrome c oxidase subunit I
- **Cellular:** Increases in cellular proliferation and decreases in apoptosis (anti-apoptotic protein Bc1-x_L)
- **Genomics/Epigenetics:** Differentially expressed genes using microarray analysis (cyclooxygenase 2, osteopontin, IGF-2, TGFα), hypermethylation of MGMT, etc.
- **Proteomics:** Up-regulation and down-regulation of specific proteins (EB1, Pms2 and or ERCC1)
- **Micro-vascular:** expression of pre-angiogenic proteins (COX-2, iNOS, VEGF)

While a plethora of potential biomarkers have been identified, few studies have attempted to validate the sensitivities and specificities of these biomarkers for use in a clinical setting. There are many reasons for the lag from discovery of biomarkers to use their clinical usage. One is the validation of novel biomarkers requires obtaining a large cohort of patients and most studies have small sample sizes. Validation of novel biomarkers also requires its study in all stages of cancer formation, specifically in pre-malignant growth. Finally, most cancers progress through multiple carcinogenic

pathways which makes finding a biomarkers specific mechanism of action difficult to assess. Since CRC encompasses multiple phenotypes, it is widely accepted that multiple biomarkers are needed to aid in early detection and risk stratification of colorectal and other cancers through field carcinogenesis.

1.4 Cohesin as a Biomarker

Recent research has validated that chromosomal instability (CIN) plays a major role in early CRC development (Barber et al., 2008). Chromosomal instability results in the loss or gain of entire or large regions of chromosomes, however the exact mechanism of action is poorly understood. This loss or gain in chromosomes could result in the removal of tumor suppressor genes or the gain of proto-oncogenes in a cell and consequentially cause abnormal growth and tumorigenesis. Recent studies have tried to determine whether whole chromosome missegregation results from errors in anaphase of mitosis (including defects in spindle assembly checkpoints, sister chromatid cohesion, kinetochore-microtubule (kMT) attachment, and chromosome number leading to lagging chromosomes or pre-mitotic replication stress generating partially replicated chromosomes (Bakhoum et al., 2014). Results from a study analyzing the differences between CIN+ and CIN- cells found that the most frequent difference between the two cell lines was the presence of lagging chromosomes during anaphase (Bakoum et al. 2014). The study also showed that repression of mitotic defects leading to lagging strands significantly represses CIN+ cells. If these mitotic defects can be targeted, it would enable future studies to identify the causes of early CRC development.

Recent studies have suggested that the cohesin family of proteins, which are essential in the chromosomal segregation process, could play a large factor in CIN. Cohesin is a multimeric complex made of structural maintenance proteins (SMC1 and SMC3), sister chromatid cohesion proteins (SA-1 and SA-2), and the chromatin loader (NIPBL) with the overall function of holding sister chromatids together from the time of replication in S phase until their separation in anaphase to ensure proper chromosome segregation during mitosis (Fig. 2). The SMC proteins are large polypeptides ranging from 1,000-1,300 amino acids with molecular weights ranging from 110-170kDa (Mannini et al., 2009). The ability of two SMC subunits to form a ring around DNA is due to the dimerization of the hinge domain that connects the subunits two coiled-coil domains. Besides its role in chromosomal segregation, cohesin proteins are emerging as a mechanism for regulating gene expression and maintenance of genome stability, which means that cohesion has many functions beyond what was first thought. Evidence of this concept comes from a study where *Drosophila* containing a mutation in cohesion loading protein NIPBL was found to be deficient in activation of homeobox genes. Additionally, cohesion has been found to regulate gene expression by interacting with the zinc-finger DNA binding protein, insulator protein CCCTC binding factor (CTCF) (Wendt et al., 2008). In addition, SMC1 and SMC3 have also been identified as having a role in DNA repair and genome stability as part of the RC-1 complex which promotes the repair of DNA gaps and deletions (Xu et al., 2011).

Observing that cohesin proteins play versatile functional roles in cellular processes, it comes as no surprise that cohesin and associated proteins have been found to

play a role in many cancer developments and other human disorders. The overexpression of the cohesion-binding protein WAPL, responsible for the timely release of cohesion from chromosome arms during prophase, has been observed in cervical cancers (Mannini et al., 2010). As well, WAPL down-regulation has been shown to inhibit the tumor growth in cervical cancer. Recent studies have found that mutations and deletions in SMC1 were found in patients suffering from Cornelia de Lange syndrome (CdLS) and in a range of tumors (Mannini et al., 2010).

Since early CRC development is marked by chromosomal instability resulting in a sequence of epigenetic and genetic mutations that cause the activation of oncogenes and inactivation of tumor suppressor genes, the finding that cohesin related genes (SMC1, NIPBL, SMC3, STAG3) are mutated in early CRC development may explain the aneuploidy and enhanced rate of loss of heterozygosis seen in tumor progression. Specifically, a total of 11 different mutations of the SMC gene have been reported in colorectal cancers (Mannini, 2010). These mutations are all either missense or small-inframe deletions that maintain the open reading frame of the gene resulting in a protein with residual function. Recognizing the role of cohesin, it is possible that these mutations might inhibit its ability to functionally load chromosomes by preventing hinge opening/dimerization or by interfering with gene expression.

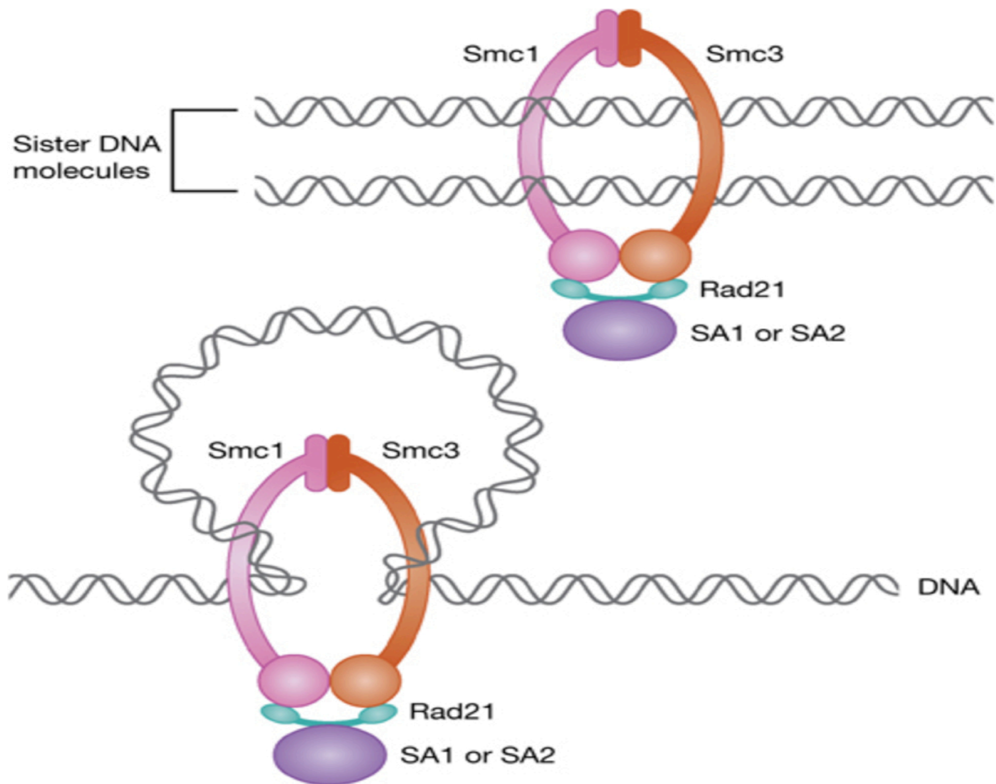

Figure 2. Structure of Cohesin Complex. Above illustrates how the cohesin subunits interact together. The SMC1 and SMC3 structural maintenance proteins dimerize and after ATP hydrolysis, they form a ring around double stranded DNA during replication. On the bottom, a representation of how cohesin might form DNA loops and affects gene transcription by acting as an insulator. Figure is a replicate from Peters et al., 2012, “The many functions of cohesin—different rings to rule them all?”

1.5 Specific aims

While mutations in genes encoding cohesin have been identified in CRC's, expression levels of these proteins and associated genes in early cancer development have not been evaluated. In order to evaluate whether cells with impaired cohesion function cause chromosomal instability leading to early events in colorectal cancer formation, the level of cohesin expression from cancer related genes needs to be assessed. Using immunohistochemical analysis, our lab has shown early evidence that cohesion proteins SA-1 and NIPBL are down regulated early in human colorectal cancer (polyp stage). More importantly, evidence also supports that this down regulation is observed in adjacent tissue due to field carcinogenesis. In order to determine cohesin's role in early colon carcinogenesis, this study also compared the gene expression of cohesion genes STAG1 (SA-1 protein) and SMC3 in human rectal biopsies to determine whether cohesion mediated chromosomal instability is a factor in early colon carcinogenesis. This data suggests that cohesion mediated chromosomal instability via epigenetic silencing could be a major pathway in early colon carcinogenesis. If this hypothesis can be verified, it would serve as a valuable biomarker for field carcinogenesis and risk assessment

II. METHODS

2.1 Experimental Design

Immunohistochemical preparation. In this translational study, we assed whether or not cohesin could be contributing to early colorectal cancer progression. Using immunohistochemical analysis, we analyzed cohesin proteins SA-1, SMC3, and NIPBL from different grades of 72 tumors, 10 tumor-adjacent histologically normal tissue, and 10 control samples (non-CRC tissue).

Patient screening. We assessed the gene expression levels of cohesin proteins SA-1 and SMC-3 in 50 human rectal mucosa samples obtained during a routine colonoscopy at the endoscopy unit at Boston Medical Center from September 2013 to March 2014. Biopsies were collected following the Institutional Review Board (IRB) guidelines after obtaining informed consent. There were 33 males (66%) and 17 females (34%). The mean ages were 56.1 years for males and 56.6 for females. Of all patients, 27% had no polyps, 25% had hyperplastic polyps, 4% had serrated adenomas, 32% had tubular adenomas, and adenocarcinomas were present in 2% of patients. Of patients with hyperplastic polyps, 75% were found in the descending (left) colon, 33% were found in the transverse colon, and 8% were found in the ascending (right) colon. Of patients with pre-cancerous polyps, 44% were found in the descending colon, 50% were found in the ascending colon, and 6% were found in the transverse colon.

Biopsy collection. Eligible patients were between 50-75 years of age and English-speaking. Patients could not have a prior history of Inflammatory Bowel Disease, a personal history of colon neoplasia (polyps and/or cancer), or a genetic history of genetic

colon cancer syndromes (i.e. familial adenomatous polyposis, hereditary non-polyposis colon cancer. The research assistant administered a 5-minute questionnaire to the patient in the pre-procedure holding area at the Boston Medical Center Endoscopy Unit. The questionnaire estimates the risk for colonic neoplasia (high, normal, low) and has been used previously in the BMC population. Patients underwent the standard colonoscopy procedure under sedation as per usual care. The size, location, and morphology of all polyps excised were documented on a data collection sheet (Appendix A) and were later correlated to the corresponding pathology report which determined whether polyps were within the normal limits, hyperplastic, adenomas, or adenocarcinomas. Using the Boston Bowel Preparation Scale, the bowel cleanliness of the patient was also recorded on a scale of 0-3 (Appendix A). The gastroenterologist, using standard flexible endoscopy biopsy forceps, obtained rectal biopsies from histologically normal-appearing mucosa. For each patient, 6 punch biopsies roughly 1 millimeter thick were obtained 10 centimeters above the anus. Biopsies were then transferred to 25mL of PBS and placed on dry ice before being stored long-term in a -80°C freezer.

2.2 Experimental Techniques and Materials

Immunohistochemistry. In order to determine which proteins to target for analysis, IHC staining was performed on human tissue arrays to analyze protein expression. Human CRC tissue arrays and CRC cell lines HT-29 (CIN+), HCT-116 (CIN-) (US Biomax, Rockville, MD) were deparaffinized and then rehydrated with xylene and graded alcohol washes. After quenching of endogenous peroxidase activity in

3% hydrogen peroxide, the slides were blocked with 5% horse serum. The slides were incubated overnight in SA-1, SMC3, or NIPBL antibody (Sigma Life Science, Prestige Antibodies, St. Louis MO) followed by incubation with the appropriate biotinylated secondary antibody. The sections were then developed using an avidin-biotin complex kit (Vector Laboratories, Burlingame, CA). The intensity scoring was scaled from low to high on a scale of 0-3 by an observer.

RNA isolation from colonic tissues. Samples stored at -80°C were thawed in an ice bath. Once thawed, the biopsies were transferred to 1.5mL tubes containing 1.5mm high impact zirconium beads (Denville Scientific Inc., NJ) and 0.5mL of TRI Reagent (Molecular Research Center Inc., Cincinnati, OH). The samples were then homogenized at room temperate using a BeadBug homogenizer (Benchmark Scientific, Edison, NJ). Total RNA was isolated from the homogenized tissue using a RiboPure Kit (Life Technologies, Woburn, MA) following the manufacturer's instructions. Homogenized tissue was added to 100µL of bromochloropropane (BCP), vortexed at maximum speed for 15 seconds, and incubated at room temperature for 5 min. The samples were centrifuged at 12,000 x g for 10 min at 4°C to separate the mixture into a lower, red, organic phase, an interphase, and a colorless, upper, aqueous phase. The aqueous phase was transferred to a new 1.5mL microcentrifuge tube followed by the addition of 200µL 100% ethanol (the organic and interphase were stored at a -80°C for later use). The samples were vortexed immediately at maximum speed for 5 seconds to avoid RNA precipitation. The samples were transferred to collection tubes with Filter Cartridges and centrifuged at 12,0000 x g for 30 seconds at room temperature until all the liquid was

through the filter. With the RNA bound to the Filter Cartridge, the flow through was discarded and 500µL of Wash Solution was added to the collection tube/Filter Cartridge assembly. The samples were centrifuged at 12,000 x g for 30 seconds at which time the flow through liquid was discarded. A second round of Wash Solution, centrifuging, and discarding of flow though, followed this. The Filter Cartridge with bound RNA was then transferred to a new collection tube. In order to elute the RNA, 45µL of Elution Buffer was added to the filter column and incubated at room temperate for 2 min. The collection tube was then centrifuged for 30 seconds at room temperate until the Elution Buffer containing RNA had flowed through the filter column. Quantification of RNA concentration was performed using the NanoDrop 2000 UV-Visiable Spectrophotometer (Thermo Scientific, Tewksbury, MA).

Complementary DNA synthesis. Complementary DNA (cDNA) synthesis was performed using 10µL of standardized RNA and 10x reverse transcriptase buffer, 25x dNTP mix (100mM), 10x Random Primers, Multiscribe reverse transcriptase, RNase inhibitor, and RNase/DNase-free water in a Verti 96-Well Thermal Cycler (Life Technologies, Woburn, MA).

Quantitative Real Time-PCR analysis. Complementary DNA for all samples was standardized to 50ng/µL after quantification using the NanoDrop 2000 UV-Visiable Spectrophotometer. Each STAG1 and SMC3 PCR reaction was standardized to 50 ng/µL by combining 4 µL of cDNA product with the TaqMan probe and PCR Mastermix (Applied Biosystems, Carlsbad, CA). Real Time-PCR was performed using a StepOne Plus Real Time-PCR System (Life Technologies, Tewksbury, MA). All samples were

normalized to β-actin and average fold differences were calculated using the comparative Ct method (Livak, 2001). The threshold of fold change significance was set as >1.5 (up-regulation) and <0.67 (down-regulation).

Table 1. Advanced Neoplastic Polyp Characterizations. The sex, age, location, size, and pathology corresponding to the rectal biopsies collected .

Patient no.	Sex	Age	Polyp location	Size (mm)	Pathology
1	F	54	Descending	7	Tubular adenoma
2	M	53	Descending	6	Tubular adenoma
3	F	51	Ascending	2	Tubular adenoma
4	M	57	Descending	5	Tubular adenoma
5	M	58	Transverse	3	Tubular adenoma
6	M	49	Descending	2	Tubular adenoma
7	M	51	Ascending/Transverse	8/20	Tubular adenoma
8	F	59	Ascending/Transverse	3/3	Tubular adenoma
9	M	53	Descending	15	Moderately differentiated adenocarcinoma
10	M	62	Ascending/ /Descending	2/13	Tubular adenoma
11	M	55	Ascending	1	Tubular adenoma
12	M	60	Descending	4	Tubular adenoma
13	M	54	Transverse	3	Tubular adenoma
14	M	54	Ascending	3	Tubular adenoma
15	M	71	Descending	3	Tubular adenoma
16	M	60	Transverse/Descending	5/3	Tubular adenoma
17	F	51	Ascending	6	Sessile serrated adenoma

III. RESULTS

3.1 SA-1 and NIPBL are down regulated in CRC

A recent proteomic study (Barber et al., 2008) found that human CRC cells carry multiple somatic mutations in genes that regulate cohesin proteins (SMC-1, NIPBL, SA-3). Also, the knock out of these genes resulted in CIN. To determine if these cohesin proteins are associated with the early development of CRC, we first examined SA-1, SA-2, SMC and NIPBL protein expression in human colon tissue specimens using immunohistochemical analysis. While 100% of control samples stained positively with SA-1 protein, that percentage dropped to 35% in cancerous samples (Fig. 4). To determine if the decrease in SA-1 expression was observed in CRC with chromosomal instability, CIN+ (HT-29) and CIN- (HCT-116) cell lines were tested for SA-1 expression. We found that SA-1 expressed a 1.4 fold decrease in cell lines that were CIN+ compared to cell lines that were CIN-. Expression levels of SA-1 and NIPBL protein were found to be significantly under expressed in adenomas, malignant CRC, and metastatic CRC ($P<0.01$) (Fig. 3). To determine if SA-1 under expression was also observable in the field of carcinogenesis, expression levels of SA-1 of histologically normal appearing mucosa adjacent to an adenoma were examined and were also down regulated by 20% (Fig. 4). The intensity scoring for SA-2 expression showed very little reactivity in control samples and the expression of SMC3 did not significantly differ between cancer and control samples.

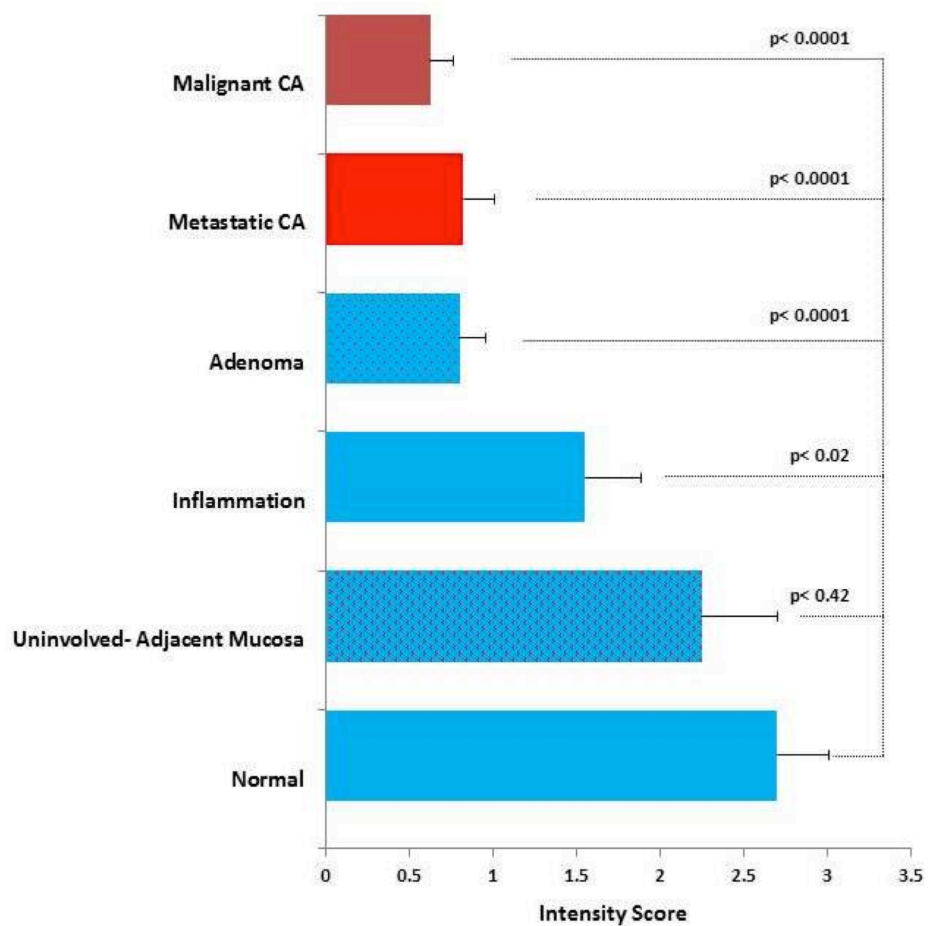

Figure 3. Immunohistochemical (IHC) analysis of the cohesin protein, SA-1, shows its expression intensity on a scale of low to high, 0 being the lowest and 3.5 being the highest. IHC was performed on human tissue assays of varying degrees of carcinogenesis, normal colonic mucosa, and uninvolved mucosa adjacent to an adenoma.

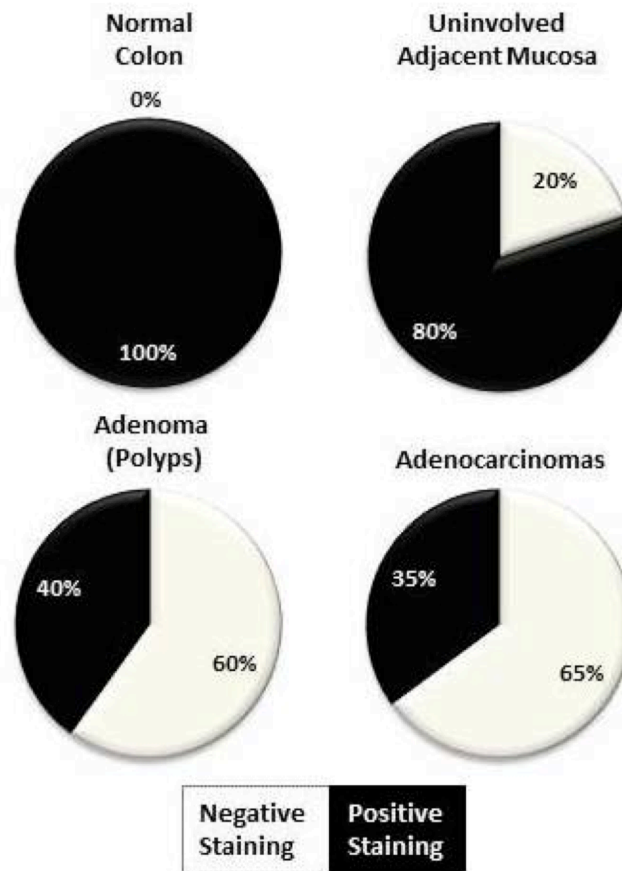

Figure 4. This figure shows the percent of positively expressed SA-1 using immunohistochemistry in normal, uninvolved mucosa adjacent to an adenoma, adenoma, and adenocarcinoma human tissue. This represents the observation that SA-1 is down regulated early in CRC cancer formation and can be observed through the field effect.

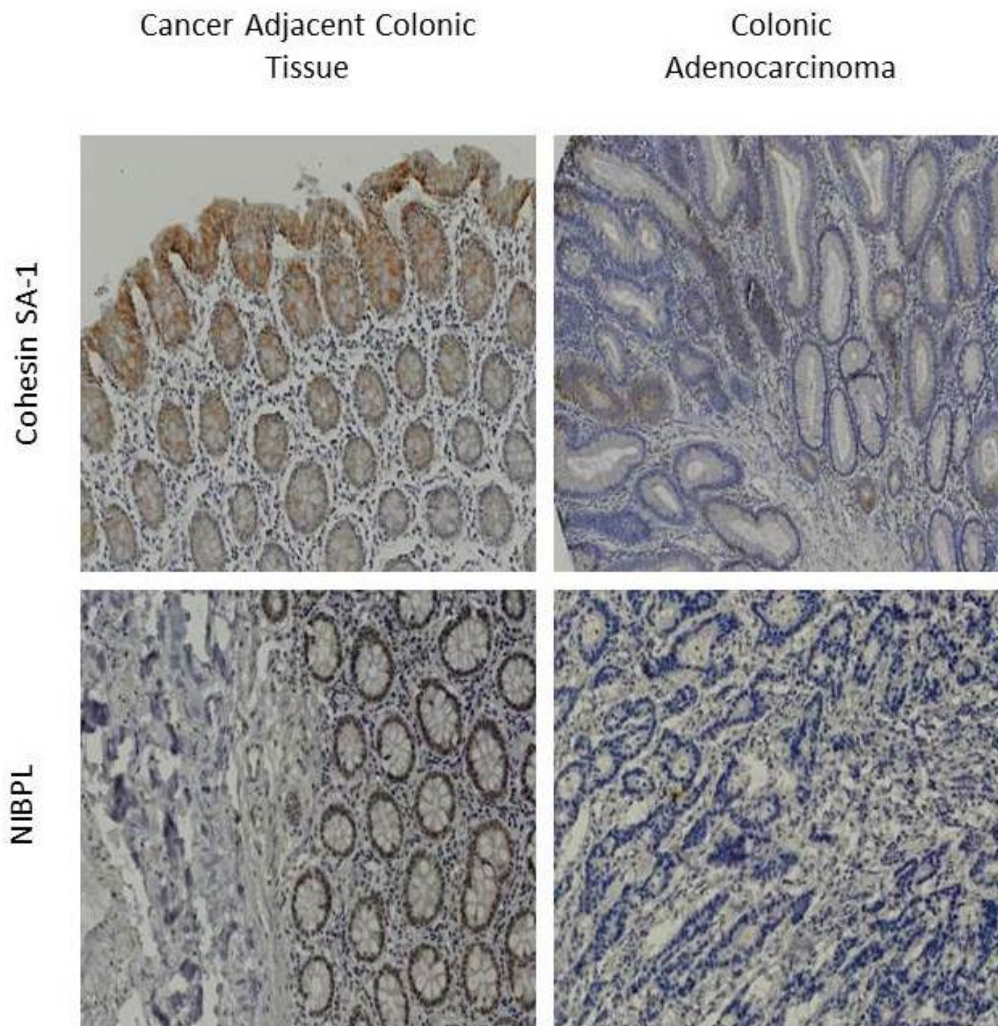

Figure 5. NIPBL and SA-1 Immunohistochemical Staining. IHC staining of SA-1 and NIPBL was done on human tissue containing colonic adenocarcinomas and the tissue adjacent to the adenocarcinoma to determine if there was a histological difference seen in the field of cancer.

3.2 SA-1 and SMC3 are up regulated in the field of cancer

In order to assess whether cohesin related genes are differentially expressed in the field of effect, the RNA expression levels of STAG1 (the gene encoding SA-1) and SMC3 from histologically normal rectal mucosa were evaluated using quantitative Real Time-PCR analysis. STAG1 was selected because of the significant results SA-1 showed from IHC analysis. While SMC3 was not significantly expressed during IHC analysis, other studies have shown that SMC3 is mutated in CRC. This along with the protein's association with gene expression, DNA repair, and sister chromatid cohesion made it a viable choice. Additionally, SMC3 has been shown to be up regulated in CRC but not in the field of the cancer. The gene expression of SA-1 and SMC3 were up regulated nearly 2 fold in the field of effect in patients harboring either hyperplastic or adenomous polyps as compared to the control (no dysplasia) (Fig. 6). The level of SA-1 expression was significantly increased in patients with hyperplastic polyps ($P<0.05$) and adenomous polyps ($P<0.05$). We then assessed whether there was difference between cohesion expression of patients with left versus right sided neoplasia and found that SMC3 expression in patients with pre-cancerous growth was significantly ($P<0.01$) overexpressed in the descending colon; nearly a 500% increase (Tab. 3).

Table 2. Percent Difference of SA-1 and SMC3 RNA Expression. Using the comparative Ct method and quantitative PCR analysis (Livak, 2001), we compared the RNA expression of cohesin proteins in human rectal biopsies between patients with neoplastic, hyperplastic, or adenomous polyps to that of patients without any polyps. Cohesin protein SA-1 was statistically overexpressed in patients harboring any type of neoplastic polyp.

* $p < 0.05$	Difference (% of control)	
	SA-1	**SMC-3**
Adenoma	96*	99
Hyperplastic	109*	97
Neoplastic	118*	98

Table 3. **Cohesin RNA Expression in Ascending and Descending Colon**. Using the comparative Ct method and quantitative PCR analysis (Livak, 2001), we compared the RNA expression of SA-1 and SMC proteins in human rectal biopsies between patients with neoplastic, hyperplastic, and adenomous polyps. SMC3 was significantly up regulated in the left, descending colon compared to the control.

*$p<0.05$	Difference (% of control)	
	SA-1	**SMC3**
Left colon	126	500*
Right colon	73	207*

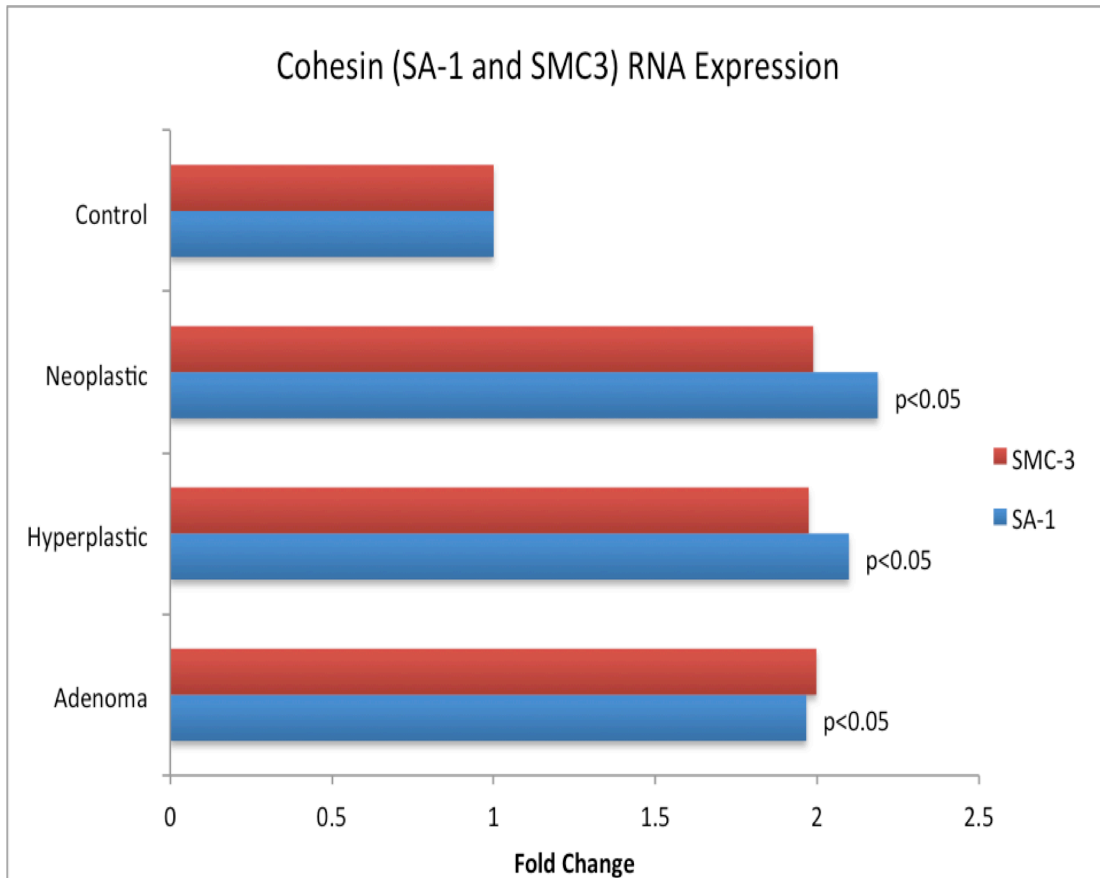

Figure 6. After quantification of SMC3 and SA-1 RNA expression using PCR-analysis, the fold change compared to control was assessed. While both SA-1 and SMC3 were overexpressed in rectal mucosal biopsies nearly 2 fold, only SA-1 was significantly up regulated in biopsies from patients with all types of polyp classifications.

IV. DISCUSSION

In the world of personalized medicine, colorectal cancer is at the forefront of treatment. Using genomic analysis, we are now able to individually screen for patients for *KRAS* mutations, which informs clinicians that the patient will show resistance towards treatment with monoclonal antibody therapy cetuximab and panitumumab, drugs which target epidermal growth factor (EGFR). Recently, assays that detect DNA methylation biomarkers (genes that are abnormally repressed due to promoter methylation observed in CRC) have become commercially available. One of these assays, ColoVantage®, is a blood based test which is able to detect the presence of methylated septin 9 with an overall sensitivity of 90%. ColoSure 500 TM is also a commercially available fecal-based assay which detects methylation of the vimentin gene. In combination with a colonoscopy, this dual screening approach can reach a sensitivity of 88% (Hudler et al., 2014). These and other assays currently undergoing clinical trials show that novel biomarkers are slowly becoming accepted as the standard in cancer risk stratification and detection. Novel nanotechnology and optical based sensors are allowing researchers to measure panels of specific cancer biomarkers with clinical implications.

Using immunohistochemical and PCR analysis, we were able to show that not only are specific cohesin proteins differentially expressed at the genetic and protein level in pre-cancerous and cancerous samples, but these changes are also observable in adjacent tissue that is histologically normal. This supports prior studies that the field effect is a useful means of determining the presence of genetic and epigenetic changes throughout the colonic mucosa. Using immunohistochemical analysis, we found that the

SA-1 protein was significantly under expressed in human CRC tissue and showed a decrease in expression in adjacent, uninvolved tissue. In CIN+ cell lines, SA-1 expression was also decreased. This data supports the hypothesis that the SA-1 protein is down regulated in CRC and that it's under expression might be causing chromosomal instability, creating a fertile zone throughout the mucosa for neoplastic growth. In accordance with other studies, SA-1 deficiency has found to be associated with aneuploidy and an increase in early tumourigenesis (Remeserio et al., 2012). Unlike SA-2, which has been found to be critical for centromeric cohesion, this study showed that SA-1 has a specific role in telomere cohesion. This evidence suggests that a deficiency in SA-1 leads to fragile telomeres and prevents them from completely replicating (Fig. 7). This defect has been shown in mouse models to cause lagging chromosomes, which is consistent with Bachoum et al. that lagging chromosomes are a signature feature of CIN.

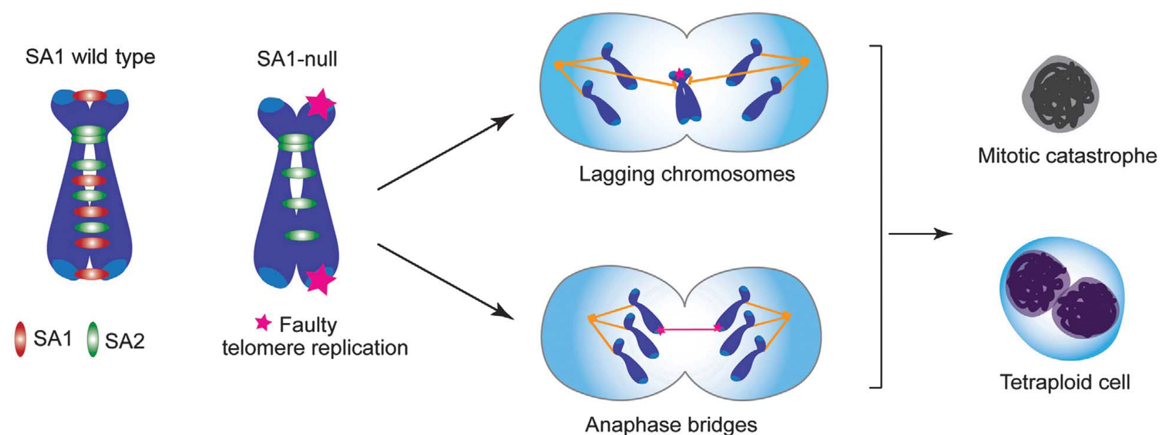

Figure 7. SA-1 Deficiency and Impaired Telomere Function. In SA-1 null cells, there is a lack of telomere cohesion leading to incomplete telomere replication and lagging chromosomes with the eventual cause of aneuploidy or cell death (Remeseiro et al. 2012)

Also of interest is the recent finding that SA-1 might play a large role in gene regulation. Cohesin SA-1 has been shown to localize at gene-associated regions of chromatin loops in the interphase nucleus, specifically promoter regions and replication origins (Cuadrado et al., 2012). Chromatin loops, one of the higher-order structures of genomic DNA, provide a suitable environment for processes such as DNA replication, transcription and repair. In cells that lack SA-1, the cohesin complex was found to be much less likely to accumulate at promoters and replication origins with the chromatin insulator CTCF. The absence of cohesin and CTCF at promoters could alter the chromatin architecture and transcription of oncogenes and tumor suppressors, which was found to be the case in CdLS. Also, down-regulation of SA-1 at replication origins could slow down replication by limiting the number of active replication units and increasing the length of chromatin loops. The results from this study add dimension to prior studies on SA-1 by showing that SA-1 is not only under expressed in animal models, but also in human CRC.

The analysis of SA-1 protein expression differed from RNA expression of the STAG1 gene, which encodes the SA-1 protein. The results showed an increase in STAG1 expression in rectal biopsies of patients who harbored adenomas. While multiple studies have identified an over expression of STAG1 in prostate, ovarian, and breast cancer (Giannini et al., 2003), it has not been reported in colorectal cancer. Research has shown that STAG1 is activated by the epidermal growth factor (EGF) pathway and plays a role in increased apoptosis. While this may be the mechanism STAG1 plays in the development of tumor formation, more research needs to be performed to validate this.

The results from this study identify that expression of the genetic/protein expression of STAG1 and SA-1 change throughout the course of cancer development. However, this could be to different lines of cancer development such as chromosomal instability, microsatellite instability, CpG island methylation and other genetic and epigenetic factors. Further research looking at the direct STAG1 pathway is needed to draw further conclusions.

The finding that SMC3 was up-regulated in rectal mucosa from patients harboring neoplastic polyps is in accordance with other studies that have shown overexpression of SMC3 in fibroblasts (Ghiselli and Lozzo, 2000) which observed that the increase in SMC3 expression led to cellular transformation and proliferation. It is not unexpected that overexpression of SMC3 could be an early event in CRC cancer formation. The heterodimer SMC1A-SMC3 complex is the main structural component of cohesin. Laugsch et al. (2013) was able to show that when changes in SMC3 expression occur within a cell, the other component, SMC1A, becomes highly unstable and can only weakly associate with chromatin. This could have a large affect on cohesin's role of keeping sister chromatids together during mitosis, eventually leading to an aneuploidy karyotype and chromosomal instability. The SMC1A-SMC3 complex is also a member of the recombination complex RC-1, which promotes DNA recombination. The RC-1 complex is responsible for catalyzing several recombination related processes such as the connecting of single stranded DNA into double stranded DNA and the repair of gaps/deletions in double stranded DNA. Defects in the cohesin complex could affect the RC-1 complex's ability to correctly catalyze these reactions. The SMC1A-SMC3

complex is also a component of the S phase DNA damage checkpoint, and deregulation of SMC3 could have negative consequences on its ability to repair damaged DNA. Perhaps most importantly, SMC3 overexpression has also been linked to activation of the APC/β-catenin/TCF4 pathway (Ghiselli et al., 2003). Mutations in the APC (adenomatosis polyposis coli) gene are found in more than 70% of colon adenomas and its loss of function is known to be an early event in colon cancer formation. Our data also showed that SMC3 was significantly overexpressed in the descending colon of patients harboring pre-cancerous polyps. This further supports our conclusion that cohesin contributes to CIN due to the fact that CIN implicated CRC is more common in the left, distal colon (Pritchard and Grady, 2011).

The observation that NIPBL was under expressed in CRC tissue is also consistent with other studies. It has recently been observed that cohesin might have a role in gene activation by formation a DNA loop upon activation by a mediator protein. Upon being co-activated by the mediator protein, NIPBL loads cohesin onto DNA segments and is able to physically and functionally connect the promoter and enhancer regions of different genes. A reduction in expression of NIPBL could affect its ability to functionally load cohesin onto chromatin. This could result in CIN in two different ways: (1) a non-functional NIPBL could lead to missegreation of sister chromatids during mitosis and lead to aneuploidy or (2) it could cause improper DNA loop formation and therefor lead to certain tumor suppressor genes being overexpressed or proto-oncogenes being under expressed. The observation that mutated NIPBL has also been seen in

Cornelia de Lange syndrome as well as CRC suggests that NIPBL could possibly be a tumor suppressor gene (Kim et al., 2013).

Since CRC with chromosomal instability is the most aggressive form of CRC, with a poor prognosis in 85%, this study represents a step forward in understanding how CIN develops. The finding that of 102 mutated genes related to CIN in colorectal cancer, 25% were cohesin-related has prompted much research on how cohesin might affect CIN (Barber et al. 2008). While our findings shed no insight on the exact role the cohesin complex plays in cancer formation, it is clear that cohesin deregulation is an early event in colorectal cancer formation. It appears that there are two main possible mechanisms of how cohesin could cause CIN: (1) mutation of cohesin proteins leads to deregulation of specific cohesin proteins and causes loss of function in cohesion's role to effectively hold sister chromatids together during replication, leading to incorrect separation of chromosomes and aneuploidy or (2) the deregulation is causing changes in chromatin architecture and negatively effecting gene expression. The results of this study have revealed that differential cohesin expression is an early event in human CRC formation, and that it could be a useful biomarker in field carcinogenesis. While this paper is one of the first to observe changes in cohesin expression at different stages of CRC, future studies are needed to further explain this observation. One drawback of this study is the small sample size of patients. A larger cohort of patients would allow a more robust analysis of cohesin expression at the hyperplastic, adenoma, and adenocarcinoma stage of CRC cancer. Also, future studies should examine cohesin expression in different phenotypes of CRC, specifically hereditary versus sporadic CRC.

The ability to identify early genetic and epigenetic events that lead to the clonal expansion of pre-neoplastic daughter cells in field effect poses as a promising future for colorectal cancer prevention. This study and studies of similar nature are answering the question of how exactly these cancer fields develop, and in doing so marks the first step towards understanding how to detect cancer before it even develops.

APPENDIX

Data Collection Sheet

Study ID ________

PLEASE TAKE 6 RECTAL PUNCH BIOPSIES:

- 10 cm above the anus
- ~1 mm thick
- Use provided forceps/jars for sample collection

Extent of exam (circle): CECUM or OTHER____________

Please list any polyps found during the exam:

Location	Bowel cleanliness (Boston Bowel Preparation Scale score)*	# of polyps found	Size(s) in mm
Right colon			#1 #2 #3 #4
Transverse colon			#1 #2 #3 #4
Left colon			#1 #2 #3 #4

*Boston Bowl Preparation Scale
3 = Colon/mucosa seen well with no residual staining/stool fragments
2 = Colon/mucosa seen well with minor residual staining/stools fragments
1 = Portion colon/colon seen due to residual staining/ stool fragments
0 = Colon/mucosa unseen due to solid stool that cannot be cleared

Appendix A. Data Collection Sheet. Collection sheet given to clinicians during routine standard of care colonoscopy to record any polyps excised and cleanliness of the bowel.

LIST OF JOURNAL ABBREVIATIONS

Am. J. Gastroenterol	American Journal of Gastroenterology
Arch Intern Med	Archives of Internal Medicine
BMJ	BMJ: British Medical Journal
BR J Cancer	British Journal of Cancer
Cancer Lett	Cancer Letters
Cancer Res	Cancer Research
Clin Genet	Clinical Genetics
Dig Dis Sci	Digestive Diseases and Sciences
Hum Mutat	Human Mutation
J. Biol. Chem	The Journal of Biological Chemistry
J. Biomed	Journal of Biomedical Science
JCO	Journal of Clinical Oncology
J Clin Pathol	Journal of Clinical Pathology
JAMA	JAMA: The Journal of the American Medical Association
Mol. Carcinog	Molecular Carcinogenesis
Natl Cancer Inst	National Cancer Institute
Nat Rev Cancer	Nature Reviews Cancer
NEJM	New England Journal of Medicine
PNAS	Proceedings of the National Academy of Sciences of the United States of America

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