MINIREVIEWS

Racial disparity in colorectal cancer: Gut microbiome and cancer stem cells

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Abstract

Over the past two decades there has been remarkable progress in cancer diagnosis, treatment and screening. The basic mechanisms leading to pathogenesis of various types of cancers are also understood better and some patients, if diagnosed at a particular stage go on to lead a normal pre-diagnosis life. Despite these achievements, racial disparity in some cancers remains a mystery. The higher incidence, aggressiveness and mortality of breast, prostate and colorectal cancers (CRCs) in African-Americans as compared to Caucasian-Americans are now well documented. The polyp-carcinoma sequence in CRC and easy access to colonic epithelia or colonic epithelial cells through colonoscopy/colonic effluent provides the opportunity to study colonic stem cells early in course of natural history of the disease. With the advent of metagenomic sequencing, uncultivable organisms can now be identified in stool and their numbers correlated with the effects on colonic epithelia. It would be expected that these techniques would revolutionize our understanding of the racial disparity in CRC and pave a way for the same in other cancers as well. Unfortunately, this has not happened. Our understanding of the underlying factors responsible in African-Americans for higher incidence and mortality from colorectal carcinoma remains minimal. In this review, we aim to summarize the available data on role of microbiome and cancer stem cells in racial disparity in CRC. This will provide a platform for further research on this topic.

Core tip: The role of microbial dysbiosis and cancer stem cells (CSCs) in colorectal cancer (CRC) has been studied extensively, however, their implication in racial disparity is not well known. A number of recent studies have shown that different dietary patterns affect gut microbiome. Likewise, dietary patterns also affect intracellular regulatory events which may affect the function of CSCs. Our objective is to consolidate the available data, on the role of gut microbiome and CSCs in racial disparity in CRC, explore a link between them and lay a foundation for further advances.

INTRODUCTION

Colorectal cancer (CRC) remains the second leading cause of cancer related mortality in the United States. However, the incidence and mortality of colon cancer is different among various racial and ethnic groups. African Americans (AAs) share the largest burden of CRC in the United States. Data from Surveillance, Epidemiology and End Results (SEER) revealed that the age-adjusted incidence of CRC in AAs, based on cases diagnosed between 2008 and 2012 was 52.3 per 100000 for men and women combined per year, compared to 41.5 per 100000 for men and women combined per year among Caucasian Americans (CAs). Similarly, the age-adjusted mortality from CRC in AAs, based on cases diagnosed between 2008 and 2012 was 21.4 per 100000 for men and women per year, compared to 15 per 100000 for men and women per year in Whites/CAs[1]. AAs not only tend to be diagnosed at a younger age but also have a worse prognosis than CAs[2,3]. Many genetic, epigenetic and environmental factors have been reported that are responsible for this racial disparity.

In recent years, there has been an increased focus on differences between microbiota of colon of healthy individuals and of patients with CRC. A relationship between microbial dysbiosis and CRC is now established[4-7].

The concept that pluripotent and self-renewing cancer stem cells (CSCs) have a pivotal role in the development and progression of many malignancies, including CRC is now well accepted. We have reported a higher proportion of CSCs (specifically CD44+ CD166- phenotype) in AAs, who also had a significantly higher

number of adenomas, compared to CAs[8]. However, underlying regulatory mechanisms remain unknown.

Recent studies have shown that host can alter gut microbiota through external (diet, obesity, *etc.*) and internal factors (microRNAs in intestinal epithelial cells)[9]. MicroRNAs (miRNAs) have also been reported to regulate CSCs[10]. Thus it is possible that the gut microbiota and CSCs are not entirely isolated domains and in AAs, the higher frequency of unfavorable mutations, through miRNAs, facilitates pathogenic microbiota over commensal bacteria (Figure 1).

DIETARY REGULATION OF MICROBIOTA AND RACIAL DISPARITY IN CRC

Human gut is a major harbinger of a wide variety of microbial cells containing approximately 10^{14} cells estimating 1000 species. The dominant composition is bacteria with 90% of species belonging to Firmicutes and Bacteroides[11]. These bacteria are in a symbiotic relationship with the intestine, utilizing undigested nutrients as substrates and in return produce various vitamins, amino acids, transform bile salts and assist in maintenance of intestinal barrier, appropriate immune response against pathogens[12]. This homeostasis is altered in a state of dysbiosis, which is overgrowth of pathogenic bacteria that are normally inhibited by commensal bacteria.

Numerous studies have been performed to examine whether and to what extent the dietary changes may affect gut microbiota. In general, these studies suggest that changes in diet and their interaction with gut microbiota exert profound effects on intestinal homeostasis through various metabolites[13]. Emergence of metagenomic sequencing has enabled identification of microorganisms not possible with 16S ribosomal RNA gene (*rRNA*) sequence-based methods and traditional culture methods[13-15]. Collective genomes of the members of a microbial community are analyzed against widely available microbial databases, thus allowing identifying microbial communities, which are virtually uncultivable. This has led to discovery of hundreds of microbial genus not previously known to exist in the human gut.

Qin *et al*[11] published a comprehensive catalogue of human gut microbial genes in 2010. Among the various conclusions, one was that *Fusobacterium* genus is not an abundant constituent of the normal gut microbiota. It is a genus of anaerobic gram-negative bacilli and has been known to cause periodontal disease. *Fusobacterium* species esp. *F. nucleatum* has been isolated from colon and fecal samples of patients with CRC in multiple studies[16-18]. Castellarin *et al*[19] even found a positive association between *Fusobacterium* and lymph node metastases.

Gao *et al*[4] examined microbiota from cancerous tissues of CRC patients and found a significant abundance of *Firmicutes* and *Fusobacteria* compared to
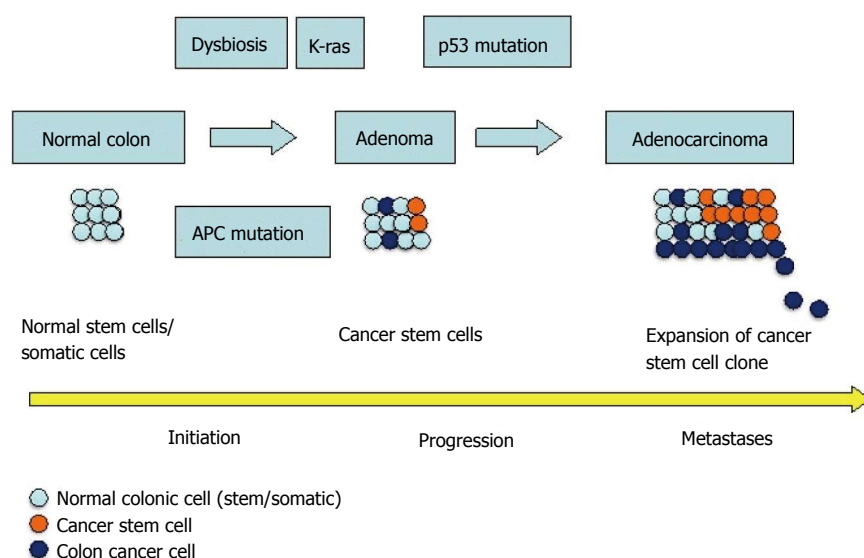
Figure 1 Cancer stem cells during development and progression of colorectal cancer. Schematic representation of the role of dysbiosis caused by microbiome alterations and accumulation of mutations in colonic stem cells leading to development and progression of colorectal cancer. APC: Adenomatous polyposis coli.

healthy individuals. Interestingly they also found that *Proteobacteria* was less abundant in patients with CRC. In the first large series sequencing of stool samples, Sobhani *et al*[5] reported that Bacteroides/Prevotella were markedly increased in patients with CRC.

Dietary components like vegetables, fiber, vitamin D are shown to be associated with a lower risk of colon cancer whereas red meat and a diet rich in saturated animal fat has been shown to be responsible for an increased risk of colon cancer[20,21]. Two major biotransformation pathways for dietary components mediated by microbiome have been reported.

A diet rich in fiber stimulates saccharolytic fermentation and production of short chain fatty acids (SCFAs) namely butyrate, acetate and propionate. These metabolites, particularly butyrate have anti-inflammatory, anti-proliferative and antineoplastic properties, while a fat rich diet stimulates the synthesis and release of bile acids in the gut[22,23].

In their study involving four racial groups, Hester *et al*[24] found that SCFA level was lower in stool from African-Americans than other racial groups. Interestingly, they also found a decreased prevalence of bacteria of *Lachnospiraceae* family in stool from African-American patients. Bacteria of *Lachnospiraceae* family have been previously shown to be associated with butyrate production in colon tissue[25]. A summary depicting bacteria, whose presence has been shown to have or probably has a positive or negative association with colon cancer in AAs has been shown in Table 1.

It has been widely reported that the higher amount of butyrate is seen in stool of healthy controls than CRC patients[26]. On the other hand secondary bile acids have been postulated to have a carcinogenic role[27].

Ou *et al*[28] examined stool from healthy AAs and from age and sex-matched native Africans and found a higher concentration of fecal secondary bile acid in AAs and a higher concentration of short-chain fatty acids in native Africans. Although the reason(s) for these increases are not known, it is possible that changes in

dietary habits are responsible for these differences.

Majority of the primary bile acids are returned to the liver by the enterohepatic circulation. A fraction of the primary bile acids escapes the enterohepatic circulation and reaches the colon. In the colon, 7-DHC (Dehydrocholesterol), converts primary bile acids into secondary bile acids, like deoxycholic acid and lithocholic acid. There is plenty of evidence to suggest that when exposed to high levels of bile acids, gastrointestinal cells undergo oxidative and nitrosative stress leading to anti-apoptotic and mutagenic properties[29]. De Boever *et al*[30] demonstrated the protective effect of *Lactobacillus* against bile salt cytotoxicity. Many studies have found that African-Americans have a lower prevalence of *Lactobacillus*, compared to other racial groups[31,32].

Moore and Moore[31] studied the stool microbial composition in populations with higher (CAs, patients with polyp) and lower CRC risk (South African blacks, native Japanese). They found a positive association of *Bacteroides* and *Bifidobacterium*, and a negative association of *Lactobacillus* and *Eubacterium aerofaciens*, with colon cancer risk.

These studies provide ample evidence that a variation in microbial composition between ethnic groups may partly be responsible in colorectal carcinogenesis and that diet plays a role in this microbial diversity.

CSCS AND RACIAL DISPARITY IN CRC

CSCs

According to the stem cell model of carcinogenesis, only some cells in a tissue possess the ability to initiate and sustain tumor growth. These cells, characterized as CSCs have two important properties: Indefinite proliferation and pleuripotency (ability to differentiate itself into more than one cell lineage)[33]. The critical role of CSCs in initiation, development and progression of CRC is now well established[34]. Mutations in normal stem, progenitor or terminally differentiated cells, are believed to be responsible for origin of CSCs, but the

Table 1 Depicting bacterial genus/families, whose presence has been shown to have or probably has a positive or negative association with colon cancer in African-Americans	
Positive association	**Negative association**
Fusobacterium	Lactobacillus
Firmicutes	Lachnospiracea
Bacteroides	Eubacterium
Bifidobacterium	

processes responsible are not completely known.

Colon stem cells are believed to exist as undifferentiated cells at the base of the crypt of lieberkuhn in the proliferative zone. The undifferentiated cells differentiate in to specialized cells as they move up the crypt-villic axis towards the luminal surface. It is estimated that, in human adults in every square centimeter of colon, there are about 14000 crypts and at a given rate of 5 d for colonic epithelium renewal; over 6×10^{14} colonocytes are produced during the individual lifetime[35,36]. The lifelong proliferation of the stem cells makes them more prone to accumulation of mutations than other short-lived cells.

Various pathways tightly regulate the processes involved in maintenance of a normal intestinal epithelium. The central among those is the canonical Wnt pathway. Canonical Wnt signals are transduced through an interaction with Frizzled family receptors (Fz) and LRP5/LRP6 (low-density lipid receptor) co-receptor to the β-catenin signaling pathway. In the absence of Wnt signaling, β-catenin becomes a part of a multiprotein degradation complex, containing tumor suppressor gene product adenomatous polyposis coli (APC), scaffold protein Axin and is phosphorylated by casein kinase Iα and glycogen synthase kinase 3β, and then ubiquinated for subsequent proteosome degradation.

In the presence of Wnt signaling, after signal transduction, Axin is recruited to cell membrane by a Fz-Disheveled (DVL) or LRP5/6 interaction. This leads to degradation of the degradation complex described above and β-catenin buildup in the nucleus. This stable nonphosphorylated β-catenin complexes with several factors and leads to activation of the transcription of several genes like *c-Myc*, *CD44*, *CCND1*, essential for DNA replication, cell cycle control and altered mitosis[37,38].

Characterization of CSCs: Identification of CSCs is a challenging task given the complexity of the cell surface markers, and their difference between various tissues, apart from the technical issues involved. One of the methods used to identify CSC is by the cell surface markers, also known as epitopes. Colonosphere formation, a functional assay is also used to characterize CSCs.

Colon CSCs have been identified by expression of numerous surface epitopes CD133, CD24, CD44, CD166, EPCAM (epithelial specific antigen/ESA), *etc*[39]. CD166 expression has been linked with shortened survival[40]. Similarly, CD44's role in tumor invasiveness

and progression has prompted it to be described as a potential CSC marker for CRC[41]. It has been shown that CSCs form tumors in SCID mice at much-diluted concentrations, which histologically resemble the primary tumor[42].

We studied the role of CD44, CD166 and ESA in CRC and reported their expression in premalignant adenomatous polyp[43] and also showed an age dependent increase in their expression, suggesting their role in tumor development and progression[43].

We have also recently observed that CD44+CD166- cell proportion in the colonic effluent as well as in the colonic mucosal cells is significantly increased in AAs with adenomas than CAs. We also observed that the colonic effluent from high risk AAs (more than 3 adenomas) contained markedly higher proportion of CD44+ CD166- cells than low risk AAs (subjects with no adenomas). We were not able to duplicate these results in colonic effluent from white population[8]. Taken together, the above observations point towards the substantial role of CSCs, not only in higher incidence, but also progression of CRC in AAs.

Racial disparity in miRNAs and signaling pathways regulating CSCs: According to the well-accepted Fearon and Vogelstein model of CRC progression, development of CRC is an outcome of accumulation of mutations in tumor suppressor genes, oncogenes; and accumulation of changes is more important than the sequence of changes[44]. This is also the basis of "adenoma-carcinoma model" in which *APC* gene mutation initiates the sporadic CRC, which accounts for 80%-85% CRC, followed by mutations in other genes-notably *K-ras*, deleted in colorectal carcinoma and *p53*[45]. Mutated *APC*, in association with β-catenin up regulates many oncogenes, notably CCND1 and c-myc[46,47]. We have recently reported that AAs had higher (48%) number of adenomas, recorded during colonoscopy, than CAs[8]. These findings confirm the data in separate studies by Corley *et al*[48] and Lebwohl *et al*[49]. In line with the Fearon and Vogelstein model, one of the reasons, in AAs, for a higher incidence of CRC could be the higher number of adenomas in them.

We also examined the rates of mutation of APC and β-catenin, in a small cohort of AAs ($n = 10$) and CAs ($n = 10$), The agarose gel electrophoresis of the PCR products of wild type and mutant *APC* gene (hAPC) in colonic biopsies from 5AAs and 6 CAs without adenomas is shown in Figure 2. Out of 10 AAs, 2 showed mutation in *APC* gene, whereas none of the CAs showed mutation in the gene. Similarly, 3 AAs showed mutation in β-catenin, as compared to none of the CAs. This preliminary data clearly supports the role of APC and β-catenin mutations in higher incidence of CRC in AAs.

MicroRNAs (miRNAs) are an expansive class of small non-coding RNAs, 18-23 nucleotides long, and regulate gene expression, either by translational repression or by mRNA degradation through cleavage. MiRNAs are atypically expressed in numerous pathological states,
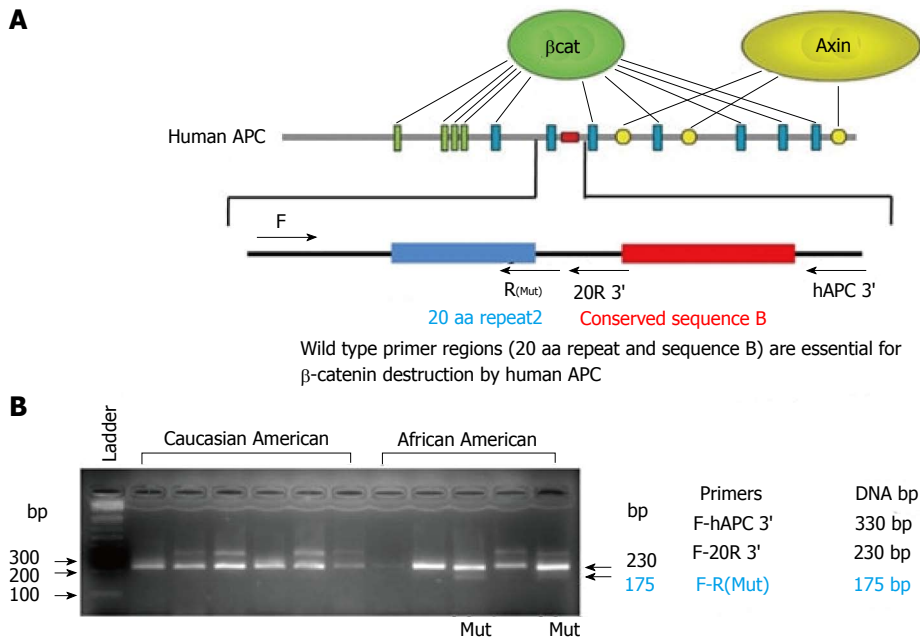
Figure 2 Schematic representation of human *APC* gene and design of appropriate primers for the wild type and mutant gene. A: Human *APC* gene with β-catenin (green and blue bars) and Axin (yellow circles) binding sites. Red bar represents conserved sequence of *APC* gene. Forward (F) and reverse (R) primers were designed to demonstrate mutation in *APC* gene; B: Agarose gel electrophoresis of PCR products showing higher rate of *APC* gene mutation (Mut: 175 bp) in the colonic mucosa of AAs without adenomas than their CA counterparts. AAs: African Americans; CA: Caucasian American; APC: Adenomatous polyposis coli.

and depending on the target, may work as oncogenes or tumor suppressors. MiRNAs' role in CRC regulation through CSCs is well researched[50-52]. We have examined the role of miRNAs 21 and 145 in regulating colon CSCs and reported that the expression of miR-21 is significantly increased and that of miR-145 markedly decreased in chemotherapy-resistant colon cancer cells, highly enriched in CSCs[53]. In colon cancer cells, forced expression of miR-145, significantly inhibits CSCs and tumor growth, whereas up-regulation of miR-21 augments the same[8]. We have also shown the role of miR-21 in age related rise of colon cancer. Upregulated miR-145 was associated with reduced levels of CD44, and β-catenin[53], both of which, we have been shown to be independently associated with racial disparity of CRC.

These observations led us to explore the role of miR-21 in ethnic differences in CRC and/or its precursor, adenoma. Ongoing studies (unpublished data) from our lab revealed that miR-21 levels in normal looking colon mucosa of AAs with adenomas were significantly higher than their CAs counterparts.

Mutation in *K-ras* gene, second most common in CRC progression, is not required for initiation of CRC. Reduced expression of miR-145 has been shown to contribute to CRC development through K-ras expression[54]. We have reported that K-RAS' lack in chemo-resistant colon cancer cells upregulates miR-145, downregulates miR-21, as well as disrupts the negative cooperation among miR-21 and miR-145[53]. Epidermal growth factor receptor (EGFR) is another transmembrane protein, whose role is well established in CRC pathogenesis. We have reported that EGFR inhibitor Cetuximab decreases miR-21 expression, suggesting another link between stem cells and definitive

mutations in CRC[55].

Mutation in *p53* gene has been shown to facilitate not only growth, but also invasiveness in colorectal adenomas. Therefore, *p53* is implicated in the adenoma-carcinoma sequence[56]. *P53* mutations have also been associated with altered miRNA processing[57].

We have recently reported a significant increase in miR-1207-5p in colonic mucosal cells cultured in stem cell media (enriched for CSCs) and CD44+CD166-cells isolated *via* flow cytometry, from AAs with adenomas. Additionally, colon cancer cell lines HCT-116 and HT-29 showed a significant increase in miR-1207-5p, compared to normal colonic epithelial cells, HCoEpiC and CCD841[8]. This lays further weight to the role of miRNAs in promoting stem cell-like properties in colon epithelial cells.

A recent whole exome sequencing study on tissues from AAs with CRC identified somatic mutations in *APC*. This also supports the role of mutations in the key protein in Wnt/β-catenin signaling pathway-APC in pathogenesis of CRC[58].

Stemness and epithelial to mesenchymal transition: A tremendous problem in management of cancer is cancer recurrence. Inspite of modern breakthroughs, in CRC, the degree of recurrence is as high as 40%-60%[59].

In any cancer, the capacity of few cells to isolate themselves from an initial site and generate a secondary tumor after implantation at a second site, defines the property of recurrence and metastases. A variety of genetic changes take place *via* a process called EMT (epithelial-mesenchymal transition) that equips CSCs to invade other tissues and survive under attachment free

conditions. In addition to mutations in *APC*, *K-RAS*, *p53* described above, activation of signaling pathways like Wnt/β-catenin, TGF-β, notch, and hedgehog is a very critical step in EMT[60,61].

The Wnt/β-catenin signaling described above regulates EMT by downstream controlling of SNAIL, TWIST, SLUG, which in turn control the expression of effectors of EMT like Vimentin, E-cadherin, and N-cadherin[62-64].

TGF-β signaling is another key pathway regulating EMT progression and is affected by activation of certain transcription factors like TWIST, SNAIL, SLUG and ZEB. In addition to activation of canonical TGF-β signaling, it is also involved in downstream activation of other canonical pathways, including Hedgehog, Notch, and Wnt and for this reason, is considered to be the master switch of the EMT process[65-67].

Notch signaling is another central mechanism for EMT development. Bao *et al*[68] demonstrated that Notch pathway increases ZEB1 expression, which leads to EMT induction by inhibiting miRNA-200. Notch expression has also been correlated with the EMT markers such as, E-cadherin and Vimentin in prostate cancer[69].

There is ample evidence to suggest that cells that undergo EMT have CSC like properties. After invading the new site, these cells initiate secondary tumor, much like CSCs[70]. The regulatory role of miRNA-200 in Notch signaling further supports the CSC theory.

Although the specific differential expression of miRNA-200 in AAs and CAs is not yet elucidated, the direct association of notch signaling with miRNA-200 inhibition, opens avenues for further investigation in the area of racial disparity (see miRNA section). We have also shown that the induced overexpression of miR-1207-5p in normal human colonic epithelial cells (HCoEpiC and CCD841) induces stemness, as well as expression of EMT markers TGF-β, CTNNB1, MMP2, Slug, Snail, and Vimentin associated with an elongated cell morphology[8], indicating its role in regulating stem cell-like properties in colon mucosal cells.

TGF-β stimulation has been shown to cause increased motility in CD133+ cells as compared to CD133- cells in non-small-cell lung carcinoma. We have discussed the differential proportion of CD44+CD166- cells in the colonic effluent as well as in colonic mucosal cells of AAs and CAs[8]. CD44 has been shown to be associated with tumor progression and metastases in CRC in various studies[71].

CONCLUSION

The conventional therapies for colon cancer do not account for CSCs. This has been postulated as one of the reasons for recurrence. It is well known that the recurrence rates are higher in AAs than CAs. In various studies, racial disparity in survival/recurrence is not well explained by differences in socioeconomic conditions, and general patterns of treatment[72-74].

It is possible that the higher rate of recurrence in AAs is in part due to prevalence of those CSCs with a less favorable mutation.

The current cytotoxic therapies act by interfering with the cell cycle of rapidly growing cells. This provides selective advantage to the slow replicating stem cells, which in fact may be enriched after chemotherapy. Data from several studies suggest a pivotal role for CSCs in regulating many malignancies, including CRC. Numerous studies have reported that CSCs or CSC like cells are highly enriched in chemotherapy resistant cancer cells. These include glioma, breast cancer, and colon cancer[75]. Results from our own studies have demonstrated that although the combined therapy of 5-FU and Oxaliplatin inhibited the growth/proliferation of human colon cancer cells (HCT-116 or HT-29), the remaining cells showed enrichment of CSCs[76].

It is well known that butyrate induces differentiation of colon cancer cells[77,78]. Forced cell differentiation has not only been shown to deplete CSCs in colon cancer but also to sensitize colon cancer cells to chemotherapy[79,80]. When these findings are viewed in light of the observations of lower amount of butyrate in stool from AAs with colon cancer than other racial groups (see section on dietary regulation of microbiota and racial disparity), it provides an interesting link between racial disparity, CSCs and CRC.

In order to successfully tackle the disparity and recurrence issues in colon cancer, a better understanding of the biological pathways is needed. Further, the focus needs to be shifted from a uniform treatment approach to a more personalized medicine. An understanding of specific CSC markers responsible for differential initiation, progression and recurrence in AAs, will help develop therapies, which target the same.

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