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Review

Bacterial biofilms as a potential contributor to mucinous colorectal cancer formation

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ABSTRACT

A prominent mucinous phenotype is observed in 10–15% of all colorectal cancers (CRCs). They are associated with a proximal location, and more commonly observed among tumors with mismatch repair defects and a promoter CpG methylator phenotype. However, none of these features has been clearly linked mechanistically to this mucinous subtype. Here, we propose that bacterial biofilms could represent a currently unappreciated contributor to mucinous CRC formation. The colonic microbiome and biofilms in particular, are emerging as important factors in tumor initiation and progression. Intriguingly, biofilms preferentially accompany proximal tumors, suggesting that there may be a direct mechanistic link with mucinous CRCs.

1. Introduction

Colorectal cancer (CRC) is the third most frequent malignancy in the world, and is the second most common cause of cancer-related mortality [1]. According to recent global cancer statistics, about 1.7 million people in the world were diagnosed with CRC, which resulted in approximately 832,000 deaths in 2015 [1]. CRC has been classified in different subtypes according to criteria such as their histological appearance. Mucinous colorectal cancer (MCC) represents an important histological subset of CRC that is observed in 10–15% of cancers, and is arbitrarily defined as a tumor with more than 50% extracellular mucin on histologic examination [2,3]. They are more commonly observed in the proximal colon [4]. Mucinous histology by itself is associated with an increase in mortality compared with their non-mucinous counterparts, even when corrected for stage [2,5]. Currently, the etiology of this subset of tumors is not yet fully understood, while they nevertheless are observed in one out of every 6–10 colorectal cancer patients. In this mini-review, we first introduce the dual character of mucus in initially preventing CRC development, while at later stages contributing to their progression. Next, we briefly describe the forms of genetic instability observed in CRC and their link to a mucinous phenotype. We then focus on the interactions between mucus, bacteria, and biofilms, and discuss probable reasons for the preferential occurrence of cancer-related biofilms in the proximal colon. Finally, we discuss the biofilm-associated mechanisms leading to enhanced mucus production during CRC initiation and development that may explain the emergence of mucinous CRCs. Many papers used for our review arbitrarily define mucinous tumors when showing more than 50% mucus and arbitrarily divide the colon in a proximal and distal part. For convenience we will adhere to these distinctions as well, but it should be realized that in reality these processes will follow a more gradual continuous model along the colonic tract [6].

2. The dual character of mucus in cancer formation

Mucins are secreted by various organs to protect the epithelium against the external environment. The colon represents a prime example, as a thick mucus layer is formed shielding the colonic epithelium from physical and chemical injury induced by food and microbes [7]. Improper functioning of the mucus layer is observed in patients with cystic fibrosis and inflammatory bowel disease (IBD), in both cases strongly contributing to the etiology of the disease [7,8]. Proper functioning of the mucus layer also decreases the chance that tumor growth is initiated, which among others is evidenced by the increased intestinal tumor predisposition of mice defective in MUC2 or ATOH1 [9–13], respectively, resulting in a strongly impaired mucus layer or complete absence of the mucin-producing goblet cells. This beneficial tumor suppressive effect is however reversed when tumors progress to malignancy. Elevated mucin levels have been associated with worse prognosis for various tumor types including those of the colon, and can contribute to tumor growth in various ways [14,15]. In mucinous cancer cells, the characteristic apical secretion of mucins typical for

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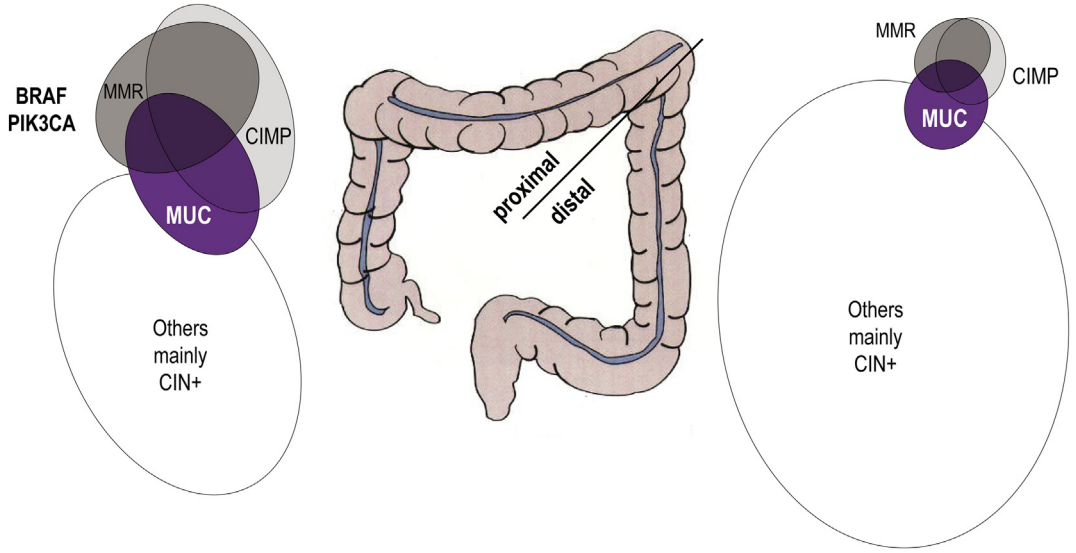

Fig. 1. Occurrence of colorectal tumors with CIN, MMR-deficiency, CIMP, and prominent mucinous phenotype in the proximal and distal colon. Tumors with this mucinous phenotype are more commonly observed in the proximal colon, and are observed in about 30–35% of lesions with MMR-deficiency and/or CIMP. They also show high levels of activating mutations in the *BRAF* and *PIK3CA* genes. Size and overlap of ellipses is in proportion to frequencies reported in the literature. CIN, Chromosomal Instability; MMR, mismatch repair; CIMP, CpG island methylator phenotype; MUC, mucinous subtype.

normal cells is lost, and the secreted mucus completely surrounds the cell surface. This has been shown to protect cancer cells from the adverse external environment and to assist cancer cells in evading immune responses [14,15]. Tumor cells also use the adhesive properties of mucins on one hand to detach from the primary tumor mass and on the other hand to attach to endothelia and invade distant structures [14]. High mucin levels have also been shown to reduce effectiveness of anticancer agents by acting as a mechanical barrier [16–18]. Thus, the mucus that initially protected the epithelial cells against tumor initiation, now supports tumor cells in their survival and growth. As such, it is important to acquire a better understanding of the mechanisms underlying mucin production in cancers.

3. Forms of genetic instability in CRC and their link to the mucinous subtype

Colorectal cancers are also categorized based on their underlying genetic instability. Chromosomal instability (CIN) accounts for more than 75% of all CRCs [19], which show a slight preference for the distal (left-sided) colorectal tract (Fig. 1). However, a prominent mucinous phenotype might be observed in only a small proportion of these tumors [20]. A second form of genetic instability observed in CRCs is DNA mismatch repair (MMR) deficiency, characterized by the accumulation of numerous mutations at the nucleotide level, especially in mono- or dinucleotide repeats. This high mutational load leads to the (in)activation of tumor-associated genes and the formation of many neo-antigens, ultimately resulting in the recruitment of abundant immune cells, a characteristic feature of this subset of tumors. These tumors account for 15% of all CRCs and predominantly arise in the proximal (right-sided) colon (70–80%) (Fig. 1) [21]. Importantly, mucinous cancers are much more prominent among this subgroup and are observed in about 30–35% of MMR-deficient lesions [22]. Lastly, a CpG island methylator phenotype (CIMP) is present in a significant subset of CRCs, resulting in hypermethylation and inactivation of promoters, some of which may be tumor suppressor genes. The mechanisms leading to CIMP are still not fully understood. On the proximal site about 30–40% of tumors are CIMP-high, whereas this is only 3–12% among distal tumors. The majority of CIMP-high tumors are characterized by a serrated histology, and are nowadays considered to represent a precursor lesion for a subset of mucinous cancers [23–26]. An extensive overlap exists between the CIMP-phenotype and CRCs with sporadic MMR inactivation due to hypermethylation of the *MLH1* promoter, one of the mismatch repair genes.

Thus, mucinous CRC is associated with a proximal location, and more commonly observed among tumors with defects in the MMR machinery and/or CIMP-phenotype (Fig. 1). In addition, they show a higher incidence among IBD patients, suggesting a link with inflammation [27]. Mucinous CRCs also show higher levels of *BRAF* and *PIK3CA* mutation than their non-mucinous counterparts [22]. However, none of these features has been clearly linked mechanistically to the mucinous subtype. Here, we propose that bacterial biofilms could represent a currently unappreciated contributor to the mucinous subtype of CRCs.

4. Mucus, bacteria, and biofilms

In the healthy colon, the secreted mucus organizes itself in a firm mucus layer directly attached to the epithelial cells, followed by a more loose layer [28]. The firm and loose mucus layers are interacted with each other and in a dynamic situation, caused by continuous mucin-degradation by microbiota and constant replenishment from goblet cells, resulting in an ascending gradient of mucus viscosity from lumen to intestinal epithelium. The firm layer is mostly reported to be devoid of bacteria, while the loose layer is inhabited by commensal bacteria that in a symbiotic fashion aid in the digestion of luminal content and exclusion of potential pathogens [28]. However, this homeostatic situation can be changed when the intestine is inflamed or temporary damaged by other insults. Under such circumstances the mucus barrier can become disrupted, allowing bacteria to come into direct contact with the epithelial cells. Bacterial species otherwise rarely observed in the healthy colon thus can find a niche to grow and possibly flourish. The chances of successful establishment are greatly increased by the formation of so-called bacterial biofilms. These are loosely defined as bacterial communities aggregating in a matrix such as mucus, which allows bacteria that normally would be rapidly purged from the colon to adhere to structures such as the colonic epithelium or tumors thereof. In these biofilms, bacterial species cooperate in various ways, as outlined in more detail in several recent reviews [29–31]. Some bacterial species are better adapted to adhering, invading or digesting the mucus layer, thereby helping others to remain in the intestine and get into

closer contact with the underlying epithelium. One example of such a cooperation is represented by fecal co-colonization of pks-positive *Escherichia coli* and enterotoxigenic *Bacteroides fragilis* [32]. The latter can reduce mucus depth allowing the pks + *E. coli* and its associated colibactin genotoxin to get into closer contact with the intestinal epithelium.

These intestinal biofilms have emerged as an important contributing factor to CRC [30,32–36]. Among others they will locally exacerbate intestinal inflammation, resulting in the production of reactive oxygen and nitrogen species that combined with genotoxic bacterial compounds, will increase the mutation rate within epithelial cells [32,33,37–39]. Other consequences are a compromised epithelial barrier function, modulation of host metabolism, and promotion of epithelial cell proliferation [30,32–34]. Combined these effects can increase the chance to trigger and promote colorectal tumorigenesis.

5. Preferential occurrence of cancer-related biofilms in the proximal colon

Initially, one bacterial strain gained special interest for CRC formation, that is *Fusobacterium nucleatum* (*Fn*). It is rarely observed in the healthy colon, but possibly from an oral source, may find a niche in the diseased colon, often in consortium with other oral bacterial species [36,40]. Nowadays, it is considered a causative agent for colorectal cancer initiation and/or progression. For example, *Fusobacterium* can increase the number of colonic tumors in the Apc^{Min} mouse model, a mouse strain that spontaneously develops intestinal tumors [41]. A potential interesting link with mucinous tumors is that several studies reported a proximal preference for *Fusobacterium* associated CRCs [35,42,43]. The same holds true for biofilms in general as they were nearly always (around 93%) detected on proximal colonic tumors, but much less frequently (about 27%) on distal tumors [33,36]. Biofilms were mostly of polymicrobial nature and frequently enriched for *B. fragilis* and oral pathogens including *Fusobacterium*. Interestingly, when a biofilm is detected on a tumor, its flanking normal tissue is mostly also covered by biofilm, suggesting that it expands over long distances in the tumor environment.

The underlying mechanisms for the preferential proximal presence of tumor-associated biofilms are still unclear. Both sides of the colon differ in various aspects such as embryonic origin and luminal content [44]. In mouse and rat the proximal mucus layer is much thinner than the distal one [45–47]. In humans the difference is less pronounced but appears to double in thickness towards the distal end [48]. A thinner proximal mucus layer possibly may be easier breached by bacteria, bringing them more readily in direct contact with the epithelial surface to form a biofilm. Secondly, the specific combination of bacteria in the proximal colon might be more efficient in forming biofilms [30,33]. It has been shown that the microbiome differs along the colorectal tract [49,50]. There is also a large degree of discordance in the microbial community compositions of intestinal mucosal samples (including tumor and paired non-tumor tissue) between biofilm-positive and biofilm-negative CRC patients [33]. A third explanation may reside in the consistency of the luminal content, which is fluidic in the proximal colon and becomes more firm towards the distal end where stool is formed. The abrasive forces of this stool may prevent an efficient formation of biofilms. In addition, during the formation of feces in the distal mouse and rat colon, most bacteria apparently become entrapped in pellets encapsulated by mucus that is captured from the epithelium [47]. Away from these pellets, the intestinal lumen and epithelium are mostly sterile. Whether bacteria also become entrapped during human stool formation remains to be determined, but in support it was shown that also human fecal pellets are enclosed by a continuous mucus gel layer [51]. Thus, the combined effects of shear force and entrapment of bacteria within the stool may prevent distal biofilm formation.

Biofilms could also be secondary to tumor formation. The emergence of tumors by itself has been shown to damage the normal mucus barrier [48], which may already facilitate biofilm formation. In the proximal colon an additional mechanism may be at play, that is about 25–30% of all proximal tumors are MMR-deficient tumors (Fig. 1). The strong local immune response that accompanies these tumors may further disrupt local tissue architecture, possibly making it easier for bacteria to find a niche. This can however not explain why virtually all proximal tumors show biofilms.

6. Biofilm-associated mechanisms leading to enhanced mucus production

Although it is not entirely clear why biofilms preferentially accompany proximal tumors, it is intriguing that this is also the side where most mucinous tumors are formed, suggesting that there may be a direct mechanistic link. So what evidence exists to support such a hypothesis? Mucus production in the colon is dynamic and can be influenced by various factors. One important direct contributor to the amount and composition of mucus secreted by the colonic cells, are bacteria [52]. For example, germ-free mice show significantly lower amounts of MUC2 protein in their colonic mucus layer, making the mucus more penetrable compared with conventionally raised mice, while gavage with cecal microbiota increases MUC2 expression and restores the impenetrable mucus in a matter of weeks [53]. Similar observations have been made for colorectal tumor cells. Direct exposure of the human mucinous CRC cell line LS174T to highly invasive *Fn* strains strongly promoted *MUC2* expression and increased expression of the pro-inflammatory cytokine tumor necrosis factor alpha (TNF-α) [54], which by itself can also enhance mucus production (see below). Likewise is mucin production increased in HT-29 colonic tumor cells by incubating them with a pathogenic *E. coli* strain or *Vibrio cholera* bacteria [55,56]. Various other reports have presented similar observations [29].

A second more indirect link with mucus production is the exacerbation of inflammation induced by biofilms. The enhanced inflammatory response leads to the generation of large amounts of cytokines, such as TNF-α, IL-22 and others, for which several reports have shown that they can increase mucus production by colonic tumor cells [54,55,57–62]. For example, prolonged TNF-α treatment of colonic tumor cells strongly increased the stability of ATOH1 protein, one of the main transcription factors regulating mucinous differentiation, thereby increasing mucus production [62].

Taken together, it seems that inflammation and bacterial biofilms in a concerted action can induce more mucus production by tumor cells. The secreted mucus in turn provides the building blocks for an efficient biofilm formation, in a way leading to a vicious circle of biofilm formation, inflammation and enhanced mucus production.

An important prerequisite however is that the genetic alterations present in CRCs still allow for sufficient differentiation towards the mucinous direction. Several reports have shown increased *MUC2* and *ATOH1* promoter methylation and inactivation in a subset of colorectal tumors, which associated with low mucus production [9,63,64]. Obviously, in tumors where this occurred, the mucus promoting features of biofilms and inflammation will have little effect on overall mucus production. Secondly, the great majority of CRCs acquire mutations in components of the Wnt/β-catenin signaling pathway resulting in aberrantly enhanced β-catenin signaling. This is mostly accomplished by inactivating mutations in the *APC* tumor suppressor gene, and to a lesser extent activating mutations of β-catenin itself or inactivating mutations in genes such as *AXIN1/AXIN2* or *RNF43* [21,65–70]. The resulting enhanced β-catenin signal imposes a crypt progenitor phenotype onto the tumor cells [71], while simultaneously reducing but importantly not entirely blocking the possibilities for differentiation. Interestingly, we and others have shown that proximal CRCs select for mutations that lead to a moderate enhancement of β-catenin signaling, while distal tumors prefer a stronger signal [21,65–67]. We have also outlined that this phenomenon likely explains the preferential proximal

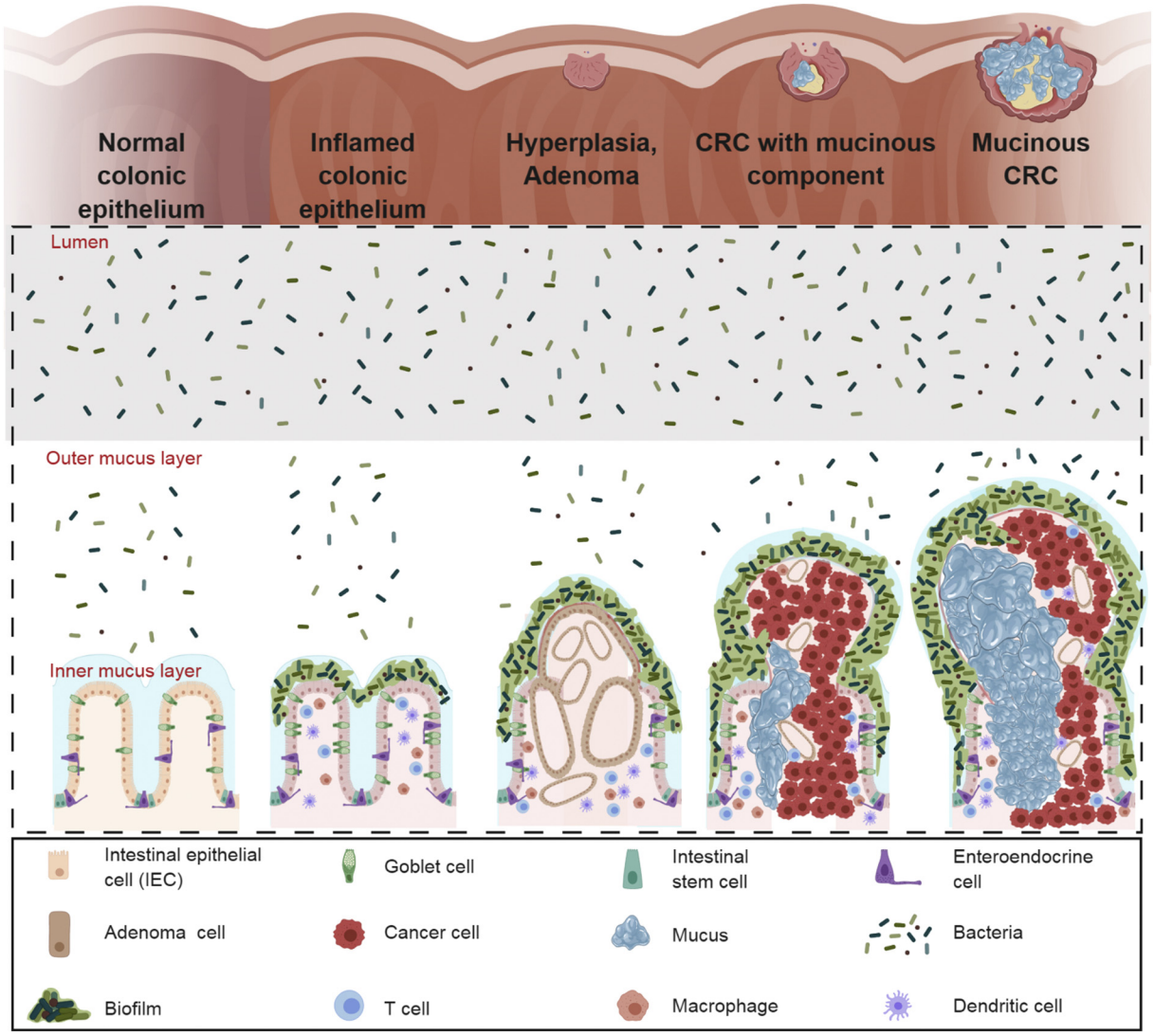

Fig. 2. Possible scenario explaining how bacterial biofilms, inflammation and colonic tumor cells may interact to form a mucinous tumor. In the healthy colon a sterile firm mucus layer separates the epithelium from a more loose mucus layer inhabited by commensal bacteria. In instances of inflammation or other insults to the epithelium the mucus barrier may become breached, possibly resulting in biofilm formation. This biofilm in concerted action with an exacerbated inflammation increase the chances of tumor initiation. Once tumors are formed, these same features can induce mucus production by tumor cells, leading to a more likely diagnosis of a mucinous CRC. Alternatively, biofilm formation is secondary to tumor initiation, as the emergence of tumors by itself damages the normal mucus barrier, thereby providing a favorable niche for bacterial colonization and subsequent biofilm formation. This figure was created with Biorender (https://biorender.com/).

location of mismatch repair deficient tumors [21]. In short, the defect in MMR leads mainly to *APC* or *CTNNB1* (encoding β-catenin protein) mutations resulting in moderate signaling levels ideal for the proximal colon, making their outgrowth on that side more likely. Whether a similar mechanism also explains the proximal preference of CIMP-high tumors remains to be determined. Anyway, the generally lower level of β-catenin signaling observed in CRCs on the proximal side likely allows for more differentiation of the tumor cells. In combination with the prevalent proximal biofilm formation and/or the accompanying inflammation, this may more readily result in tumors that generate sufficient mucus to qualify them as mucinous CRCs (more than 50% mucus).

To sum up, the following scenarios linking bacterial biofilms and mucinous CRC can be envisaged. As depicted in Fig. 2, biofilms that are enabled to form in close contact with the intestinal epithelium, for example by inflammation or other insults, can contribute to tumor initiation through the various mechanisms described above. Once the tumor is formed the bacterial biofilms in concerted action with the exacerbated inflammation, enforces more mucus production within the tumor cells. This will however only occur when the underlying genetic alterations allow for sufficient differentiation of the tumor cells or mucin gene expression. Thus in this scenario, bacterial biofilms first contribute to more tumor formation and in a second phase to a specific differentiation pattern. In a second scenario, biofilm formation is secondary to tumor initiation. In that case, the biofilms mainly contribute to tumor progression and possibly increasing mucus production, likely again in concerted action with inflammation.

7. Conclusions and perspectives

In the last decade, it is becoming increasingly clear that the colonic microbiome and bacterial biofilms play an important role in colorectal tumor development. Here, we have hypothesized that biofilms may also contribute to the specific mucinous phenotype observed in 10–15% of CRCs. This was inspired by the preferential proximal location of both mucinous CRCs as well as tumor-associated biofilms. There are however still several unanswered questions. For example, on histological examination the mucinous regions of cancers are often observed at their

invasive front, so potentially at some distance from the luminal located biofilms. This may in part represent a technical artefact, that is the pre-operative procedures to clean the patient's bowel and the subsequent fixation and paraffin embedment are likely to remove mucus that is not entrapped within tissue sections. Nevertheless, our hypothesis needs confirmation by demonstrating bacterial aggregates within reasonable distance from the mucus producing tumor cells, or providing evidence that bacterial products can affect tumor cell behavior at some distance. For the latter indirect support is already provided by the altered mucus production of normal colonic cells not being in direct contact with the luminal bacteria [53]. Furthermore, it is still unclear why biofilms mainly form in the proximal ascending colon. We have postulated some explanations, like the shear force and entrapment by stool preventing biofilms on the distal side, but whether this holds true remains to be shown. Likewise, it is unclear if the appearance and composition of biofilms associated with mucinous CRCs differs from other ones. Only few research groups have used the appropriate tools to look at biofilms and used fixation procedures that preserve mucus (e.g. Carnoy's fixative), but to our knowledge no reports have specifically looked at mucinous tumors. For the same reason it is also not known if specific bacterial strains are especially strong contributors to the mucinous subtype. Given the recent acknowledgement of biofilms contributing to colorectal tumor growth, obviously more detailed molecular and genetic analyses are needed. Moreover, as advocated by Hamada et al., this should ideally be complemented with a thorough epidemiologic analysis of lifestyle factors, dietary patterns, medications (e.g. antibiotics), and environmental exposures, which are all expected to interact with the microbiome, tumor cells and immune system [72]. In the future, these analyses may uncover potential tailor-made therapies specifically aimed at the mucinous subtype of colorectal cancers.

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