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Neoexpression of a functional primary cilium in colorectal cancer cells

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Abstract

The Hedgehog (HH) signaling pathway is involved in the maintenance of numerous cell types both during development and in the adult. Often deregulated in cancers, its involvement in colorectal cancer has come into view during the last few years, although its role remains poorly defined. In most tissues, the HH pathway is highly connected to the primary cilium (PC), an organelle that recruits functional components and regulates the HH pathway. However, normal epithelial cells of the colon display an inactive HH pathway and lack a PC. In this study, we report the presence of the PC in adenocarcinoma cells of primary colorectal tumors at all stages. Using human colorectal cancer cell lines we found a clear correlation between the presence of the PC and the expression of the final HH effector, GLI1, and provide evidence of a functional link between the two by demonstrating the recruitment of the SMO receptor to the membrane of the primary cilium. We conclude that the primary cilium directly participates in the HH pathway in colorectal cancer cells.

Keywords: Biological sciences, Cell biology

1. Introduction

The primary cilium (PC) is a solitary flagella which arises at the surface of non-proliferating cells [1]. PC have been detected in the majority of cell types, with few notable exceptions (for a complete list see: www.bowserlab.org/primarycilia/cilia3.htm) such as intestinal epithelial cells [2]. PC act as mechano- and/or chemosensors and as regulators of the cell cycle [3]. PC adopt a microtubular [9 + 0] structure, called the axoneme. Its basal body is composed of the mother centriole and its associated proteins [1]. Tubulin, which forms PC, is subject to specific post-translational modifications that are essential for intra-flagellar trafficking and ciliary motility [4]. PC structure also includes a variety of intraflagellar transport proteins that are required for the construction of the PC [5] and scaffold proteins such as tektin and ribbon proteins, which prevent spontaneous disassembly of the PC [6].

Since PC are found in most tissues, defects in ciliogenesis can be responsible for multi-organ syndromes resulting in various pathological conditions such as cystic kidney disease, mental retardation, damage to the retina, liver fibrosis, as well as malformations occurring in the cerebellum, bones and digits [7]. Studies of these diseases have led to a better understanding of the role of PC. Through calcium channels formed by the interaction of polycystins PC-1 and PC-2, PC act as mechanosensors, reacting to liquid flow in structures as various as the nephron, biliary vesicle or pancreatic duct [8]. PC organize a response based on intracellular calcium variation following which cell division can occur [9]. As PC arise from the mother centriole, they are presumed to exercise control on cell cycle and quiescence regulation [10]. They appear as post-mitotic structures which must disassemble in order for cell cycle re-entry [11]. Known cases of spermatocytes harboring a persistent PC during two meiotic divisions [12] as well as a small re-emergence of PC in G1/S and S/G2 in skin epithelial cells [13] have nevertheless to be considered [14]. The PC membrane is enriched with receptors and ionic channels and gathers together many elements of various transduction pathways in a small area, so that PC can be seen as an integrator of extracellular signals [10]. These pathways include the PDGF α response [15], the Notch pathway [16] and the non-canonical Wnt pathway [9] as well as the Hedgehog (HH) pathway [17, 18], which over the past years has become the most closely related pathway linked to PC [5, 19, 20, 21].

The HH signaling pathway is involved in cell maintenance and differentiation during development as well as in the adult [22]. Target cells harbor two receptors, Patched (PTCH) and Smoothed (SMO). PTCH captures the HH ligand, resulting in SMO recruitment and downstream activation of the HH pathway via GLI transcription factors [5, 19, 21, 23]. Three members of the family are found in mammals. The expression of GLI1, an activator of transcription, is induced by

pathway activation while GLI2 and GLI3 simply act as transcription activators or repressors [1, 19]. In both cases, processing of GLI2 and GLI3 requires targeting to the PC [10]. When the HH pathway is inactive, GLI2 and GLI3 undergo cleavage which converts them into repressors. Activation of the pathway leads SMO to be recruited to the PC membrane where it induces GLI2/3 to translocate to the nucleus and act as activators [24]. Genes regulated by the GLI transcription factors are mostly involved in the HH pathway itself (PTCH1, GLI1, HIP), cell cycle control (Cyclin D1) and stemness potential (BMI1, NANOG) [25]. In adults, the HH pathway is active in most polarized cells and participates in the control of stem and progenitor cells in many tissues (asymmetric division, self-renewal, pluripotency) [26]. Deregulation of the HH pathway is a key event in the oncogenic sequence of numerous cancers including basal cell carcinoma, small-cell lung cancer, breast cancer and pancreatic cancer [27, 28]. These cancers are characterized by an increased expression of GLI factors [29], while the link with PC is less clear. Indeed, the PC can be maintained or resorbed in tumor cells depending on the transduction pathways needed for cancer progression [30]. Data on this subject are still missing and require more investigation [31].

In colorectal cancer (CRC) cells, recent studies have shown an increase in the HH signaling pathway, including expression of various HH pathway-related components such as SHH, SMO, PTCH, GLI3 and/or GLI1 [32, 33, 34, 35, 36] but their roles in the carcinogenesis cascade remain unclear [37]. Although crucial for the HH signaling pathway, given the fact that epithelial cells of the intestine and colon are known to be non-ciliated, the possibility that PC could be expressed in colon cancer cells has not been directly explored.

In this study, we test this hypothesis by investigating the presence of the PC in colorectal cancer cells using tissue microarrays containing primary CRC at all stages matched with their resection margins as well as cancer cell lines. We found that the PC is a specific feature of CRC cells in situ being not detected in their normal epithelial counterparts. Furthermore, 3 of the 4 tested CRC cell lines displayed a PC while expressing components of the HH signaling pathway. The ability to recruit the specific HH component SMO to the PC membrane confirmed the functional link between PC and the HH signaling pathway in CRC cells.

2. Materials and methods

2.1. Human colorectal tissues

Adenoma and adenocarcinoma tissues and corresponding resection margins were obtained from 73 patients with their written informed consent according to a protocol approved by the Institutional Human Subject Review Board of the Centre Hospitalier Universitaire de Sherbrooke. Lesions were characterized by a pathologist. Adenomas were advanced adenomas showing dysplastic features.

Tumor staging was according to TMN (tumor, node, metastasis) classification (stage I to IV). Tumor grade (differentiation) was also considered (grade 1 to 4).

2.2. Antibodies

The primary antibodies used in this study were: mouse monoclonal anti-tubulin acetylated 611B-1 (Sigma-Aldrich, T6793, 1/1000), mouse monoclonal anti-polyglutamylation modification of tubulin (AdipoGen, GT335, 1/2000), rat polyclonal anti-CD49f G0H3 (BD Pharmingen, 555734, 1/5000), rabbit polyclonal anti-SMO (Abcam, ab38686, 1/500), rabbit monoclonal anti-GLI1 EPR4523 (Abcam, ab134906, 1/1000 for WB) and (Abcam, ab49314, 1/1000 for IF) and mouse monoclonal anti- β -actin (Millipore, MAB1501, 1/20,000). Preliminary results (unpublished) were obtained using rabbit polyclonal anti-GLI3C 2438B, mouse monoclonal anti-Gli3 N 6F5 and rabbit polyclonal anti-Gli3 N 2676A (Genentech) obtained courtesy of FJ de Sauvage [38]. Secondary antibodies were: AlexaFluor 488 or 594 goat anti-mouse (Molecular Probes, A11017, A11072, 1/400), goat anti-rat (Molecular Probes, A11006, A11007, 1/400), goat anti-rabbit (Molecular Probes, A11070, A11072, 1/400), ECL HRP-linked anti-mouse (GE Healthcare, NA931 V, 1/3000) and anti-rabbit (NA934 V, 1/3000).

2.3. Indirect immunofluorescence

Comparative analysis between tumors and normal tissues were performed on a Tissue Microarray (TMA) containing 65 tumors and 8 adenomas as well as their resection margins, previously characterized [39, 40]. Paraffin-embedded sections were rehydrated and antigen exposure was performed by boiling in citric acid. OCT-embedded sections were fixed in 4% PFA and processed as before [41]. Cells were seeded on cover slips and cultured 48 h in serum-free medium. Cells were fixed in 4% PFA and permeabilized in 0.1% Triton. Tissues and cells were blocked in PBS containing 5% Blotto and incubated with antibodies following the manufacturer's instructions. Detection was performed on a Leica MPS60 for paraffin sections and on a Leica DM RXA for OCT sections. Acquisitions were performed using Metamorph (Molecular Devices) software. Deconvoluted 3D pictures were obtained from Autoquant X3 (MediaCybernetics) and Imaris (Bitplan) softwares.

2.4. Cell culture

The colorectal cancer cell lines HT29, HCT116 and SW480 were originally obtained from the ATCC and used from frozen stocks at low passages. Caco-2/15, a stable clone of Caco-2 cells, has been previously described [42]. The cells were cultured as described previously [40, 43].

2.5. Western blotting analyses

Protein extractions and Western blotting were performed as described previously [44]. Briefly, cell extracts were loaded on 12% SDS-PAGE gels under denaturing conditions before being transferred onto nitrocellulose membranes.

2.6. Quantitative RT-PCR

Cells were lysed with RiboZol (Amresco). RNA extraction, reverse transcription-PCR and real-time PCR amplification were performed as previously described in an Mx3000 P qPCR system (Stratagene, La Jolla, CA) [45]. Primers used were for SHH: SHH-F: 5'-CAGTGGACATCACCACGTCT-3' and SHH-R: 5'-CTCAGGTCCTTCACCAGCTT-3'; for IHH: IHH-F: 5'-CGGCTTGACTGGGTGTATT-3' and IHH-R: 5'-CGGTCCAGGAAAATGAGCAC-3'; for PTCH1: PTCH1-F: 5'-ATCGTGAAGCCACAGAAA-3' and PTCH1-R: 5'-GCCAGAATGCCCTTCAGTAG-3'; for SMO: SMO-F: 5'-CCCAGCATGTCACCAAGATG-3' and SMO-R: 5'-GCACACCTCCTTCTCTCT-3'; for GLI1: GLI1-F: 5'-ACATCAACTCCGGCCAATAG-3' and GLI1-R: 5'-GAGGATGCTCATTCTCTGG-3'. Gene expression was established following the Pfaffl equation, using RPLPO for normalization [45]. Gene expression was expressed relative to a pool containing various CRC cell lines [46].

2.7. Statistical analysis

Independent experiments were repeated at least 3 times. Statistical analyses (ANOVA) were performed using Prism 6 (GraphPad) software.

3. Results

3.1. PC expression in human colorectal tumor biopsies

Control staining experiments were first performed on mouse kidney sections as positive control since tubule epithelial cells harbor PC [3]. Using an anti-acetylated α -tubulin antibody, ciliary shapes were clearly recognizable showing a sharp staining (Fig. 1A). No specific staining was detected using the anti-polyglutamylated tubulin antibody on these sections (Fig. 1B). We thus investigated the presence of PC in colon epithelial cells on TMA sections, which contained 65 tumors from stage I to IV and 8 adenomas, all paired with their corresponding control resection margins [39, 40] using the anti-acetylated α -tubulin antibody. As expected from previous studies showing expression of HH pathway components in the intestinal subepithelial stroma [2, 47], PC were uniformly detected in stromal cells of the lamina propria in the normal colon while epithelial cells were found to be non-ciliated (Fig. 1C). However, in cancers, PC were routinely observed in parenchymal cells (Fig. 1D). Representative high magnifications of resection

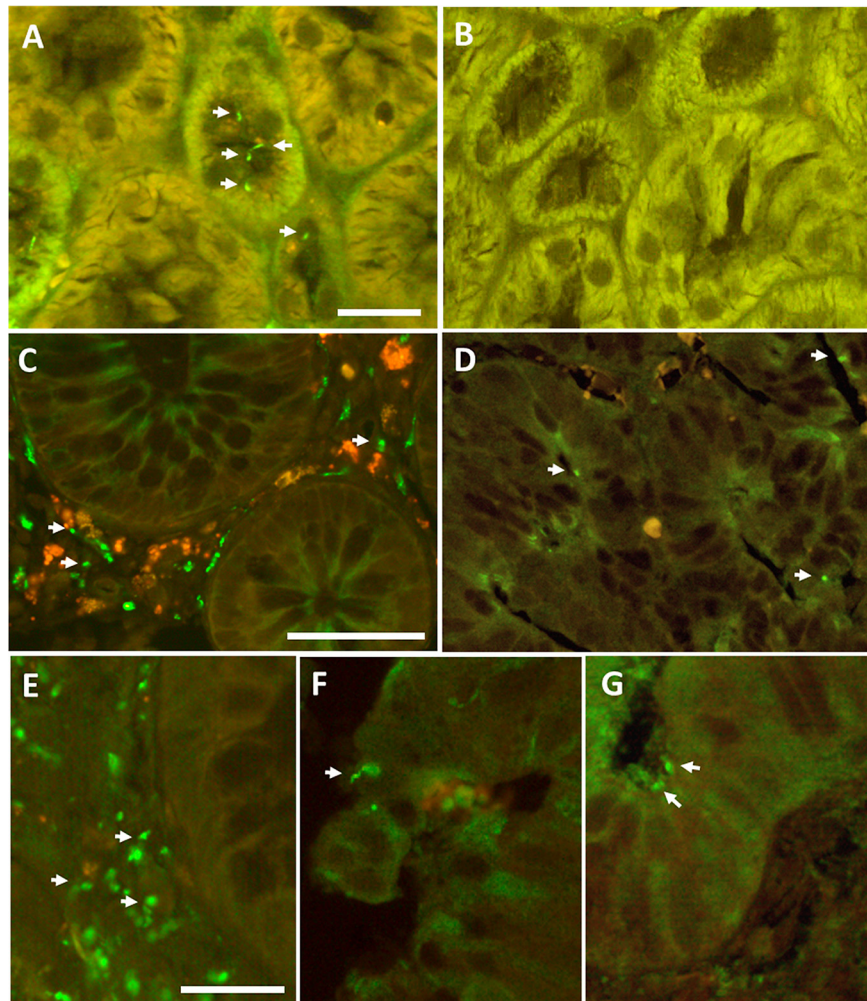


Fig. 1. Representative pictures showing immunofluorescence detection of tubulin modifications of the PC on paraffin sections. (A) PC were identified in mouse nephron epithelial cells, used as positive control, by antibodies recognizing acetylated α -tubulin (arrows). (B) No detection was observed with antibodies targeting polyglutamylation of PC. (C-G) Identification of PC in human tumors and corresponding margins from the TMA using acetylated α -tubulin antibodies. PC are absent in normal epithelial cells, but detected in the stromal cells of the resection margins (C and E: arrows) and also in tumor epithelial cells (D, F, G). Representative images of tumors were obtained from three different patients. Scales bars: 20 μ m (A-D), 50 μ m (E-G).

margin (Fig. 1E) and primary tumors (Fig. 1F,G) illustrate PC features under both conditions. Overall, PC were identified in 38 tumors (58%), 19 of which displayed a strong expression. As shown in Fig. 2, stage IV and grade 4 tumors were all, with one exception, PC positive while the detection of PC in approximately two-thirds of the low stage (CRC I and II) and low grade (CRC 2) tumors indicates that PC expression occurs relatively early in the carcinogenesis cascade but not in all lesions since advanced adenomas were devoid of PC.

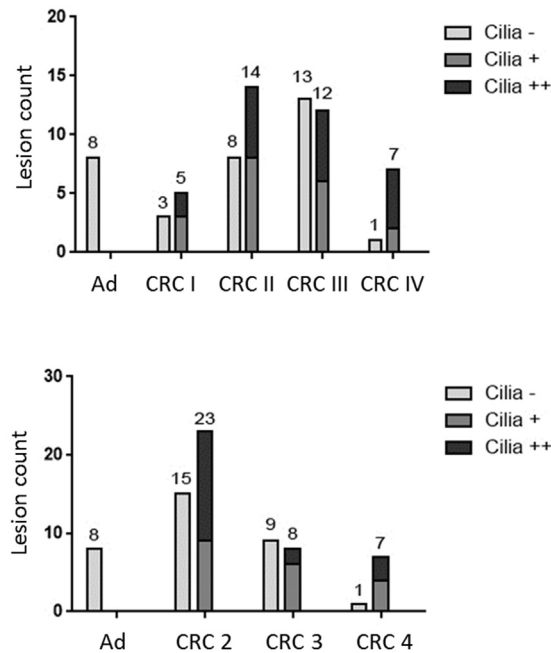


Fig. 2. Quantitative assessment of the PC in colorectal specimens. Proportions of PC positive and negative lesions were determined on TMA sections according to adenoma (Ad) and tumor stage (CRC I to IV; upper panel) or grade (CRC 2-4; lower panel). Each group was separated into three parts according to the abundance of PC in the epithelial cells (Cilia -: no cilia, Cilia +: moderate number of cilia, Cilia ++: high number of cilia). Y axis represents lesion count.

To confirm the presence of PC in primary CRC cells in situ, we performed staining on a subset of the above samples prepared in OCT compound using both acetylated α -tubulin and polyglutamylated tubulin as PC markers. To define the limit between parenchymal and stromal regions, co-staining for the $\alpha 6$ integrin subunit which predominantly localizes at the basal domain of epithelial cells [41, 48] was used. As shown in Fig. 3, PC were identified in primary CRC cells with both markers.

3.2. PC expression in colon cancer cell lines

Considering our observations in primary colorectal tumors, it can be hypothesized that at least a subset of CRC cell lines should bear PC. Using the same markers as above, we investigated the presence of PC in four colon cancer cell lines: SW480, HT29, Caco-2/15 and HCT116. As serum starvation has been showed to favor ciliogenesis [49], the cells were starved 48 h before observation.

PC-like structures were identified in three of the cell lines. As shown in Fig. 4, a structure corresponding to the classical PC shape ($\sim 5 \mu\text{m}$ long) [50] was detected in 5 to 10% of SW480 and HT29 cells. PC were also identified in HCT116 cells at a similar frequency despite the fact that they appeared shorter. Interestingly, Caco-2/15 cells were consistently found to be devoid of PC.

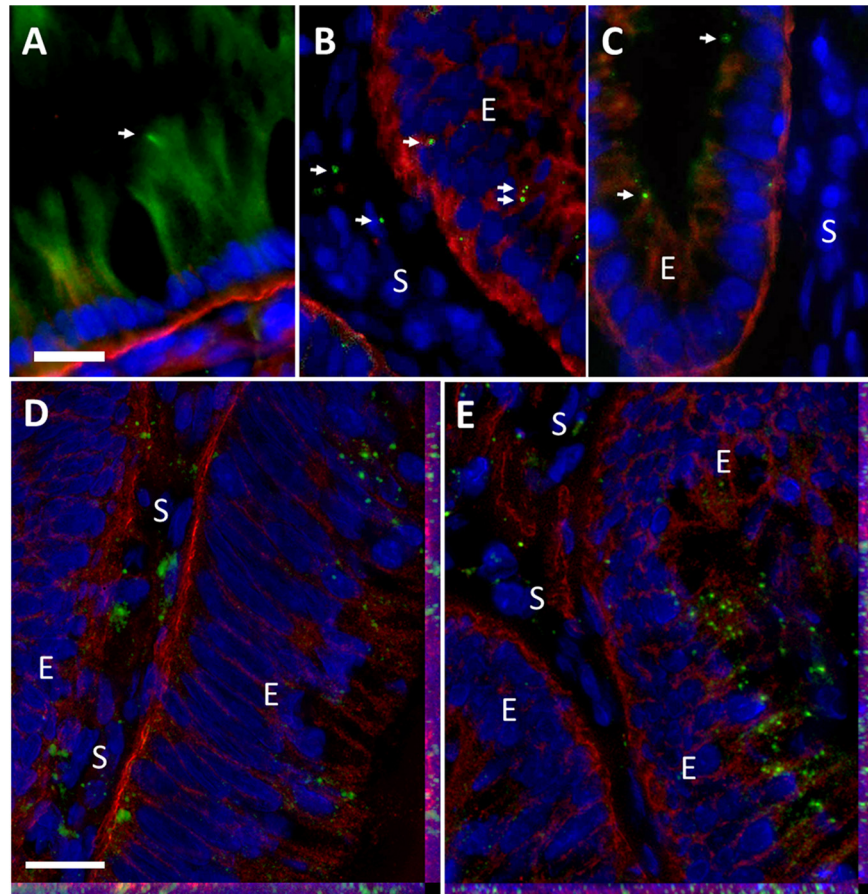


Fig. 3. Representative immunofluorescence images of the detection of PC on tumor cryosections. PC are marked with a green signal and the detection of integrin subunit $\alpha 6$ was performed to identify the base of epithelial cells (red signal). (A-C) Identification of PC using antibody detection of acetylated α -tubulin (A) and polyglutamylation (B, C) in different sections of the same tumor. PC were detected both in stromal and epithelial cells (arrows). (D-E) Deconvoluted 3D pictures showing immunoreactive acetylated α -tubulin (D) and polyglutamylated tubulin (E) in sections of a tumor from another patient. Scale bars: 100 μ m. S: Stroma; E: Epithelium.

3.3. HH pathway component expression in colon cancer cell lines

The expression of HH pathway components in colon cancer cell lines was first evaluated by RT-qPCR using a pool of colorectal cancer cell line extracts as control. As shown in Fig. 5A, transcripts for sonic HH (SHH) and Indian HH (IHH), the two ligands detected in normal intestinal epithelial cells, were found to be expressed at various levels depending on the cell line. The four cell lines also showed variable expression of PTCH and SMO receptors. Interestingly, significant levels of GLI1 expression were only detected in the PC positive cell lines HT29, HCT116 and SW480. Similarly, when tested at the protein level, significant GLI1

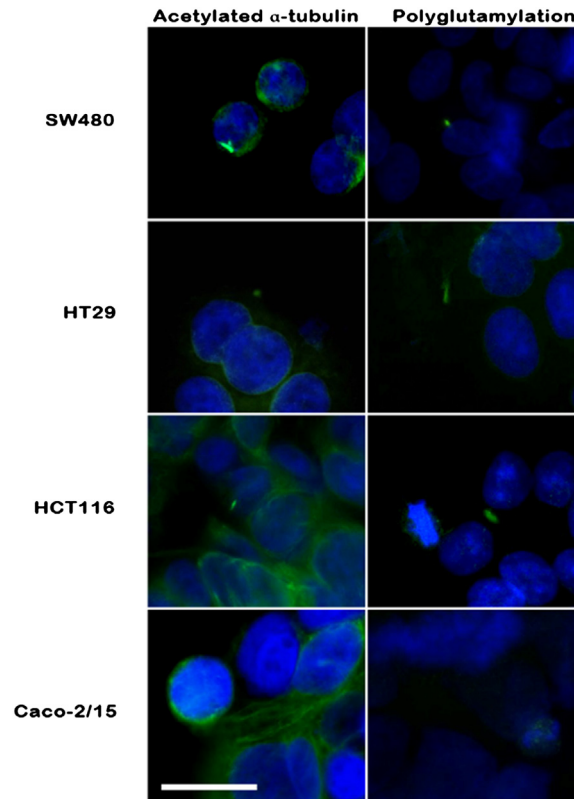


Fig. 4. Representative images showing the comparison between polyglutamylated and acetylated tubulin detections of PC in the HCT116, SW480, HT29 and Caco-2/15CRC cell lines. Column 1: acetylated α -tubulin detection of PC. Column 2: tubulin polyglutamylation detection of PC. Nuclei are stained blue with DAPI. Scale bar: 25 μ m.

expression was confirmed in HT29, HCT116 and SW480 cells while it was found to be at the limit of detection in Caco-2/15 cells (Fig. 5B).

3.4. Connection between PC and HH pathway

Dual expression of the PC with the key HH pathway component GLI1 in the HT29, HCT116 and SW480 cell lines led us to investigate more closely a potential functional link between the PC and the HH pathway, which is characterized in the canonical model by the recruitment of SMO to the PC membrane [19]. We thus assessed the expression of SMO and its localization in ciliated cells by double immunofluorescent staining approaches. As shown in Fig. 6, co-localization of SMO on the PC identified using anti-polyglutamylated tubulin antibody in SW480, HT29 and HCT116 but not in Caco-2/15 confirmed that PC recruit SMO in GLI1-expressing cells. Recruitment of SMO to the PC membrane in GLI1 expressing cells was also confirmed using an anti-acetylated tubulin antibody (Fig. 7). Taken

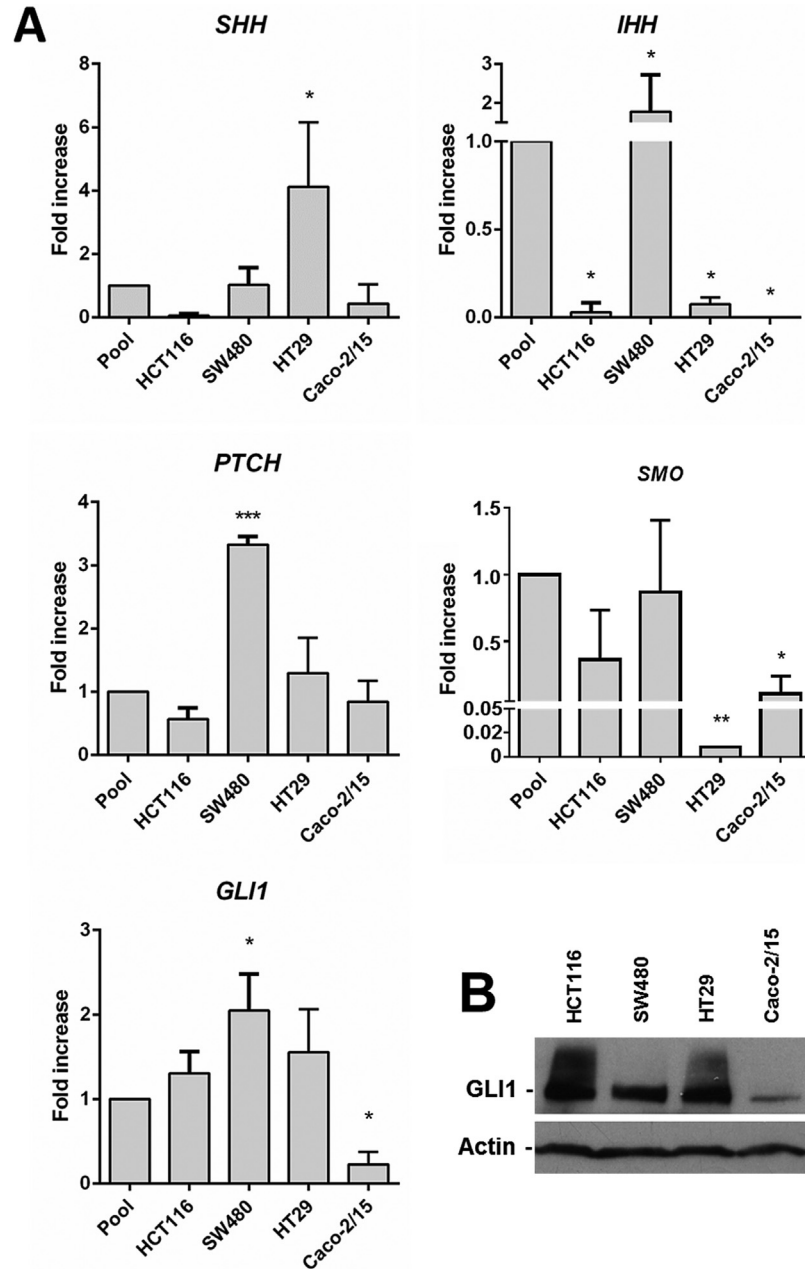


Fig. 5. Expression of different components of the HH pathway in different CRC cell lines. (A) Gene expression of SHH, IHH, PTCH, SMO and GLI1 in the CRC cell lines HCT116, SW480, HT29 and Caco-2/15. Expression of each transcript is indicated as fold change compared to its expression in a pool of colorectal cancer cell lines. qPCR was performed in triplicate and repeated three times. Results represent the mean of three independent experiments. * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$. (B) Representative Western blot showing GLI1 protein expression in the CRC cell lines HCT116, SW480, HT29 and Caco-2/15. β -Actin was used as a loading control. Full, non-adjusted images of GLI1 and actin Western blot detection are provided as supplementary material.

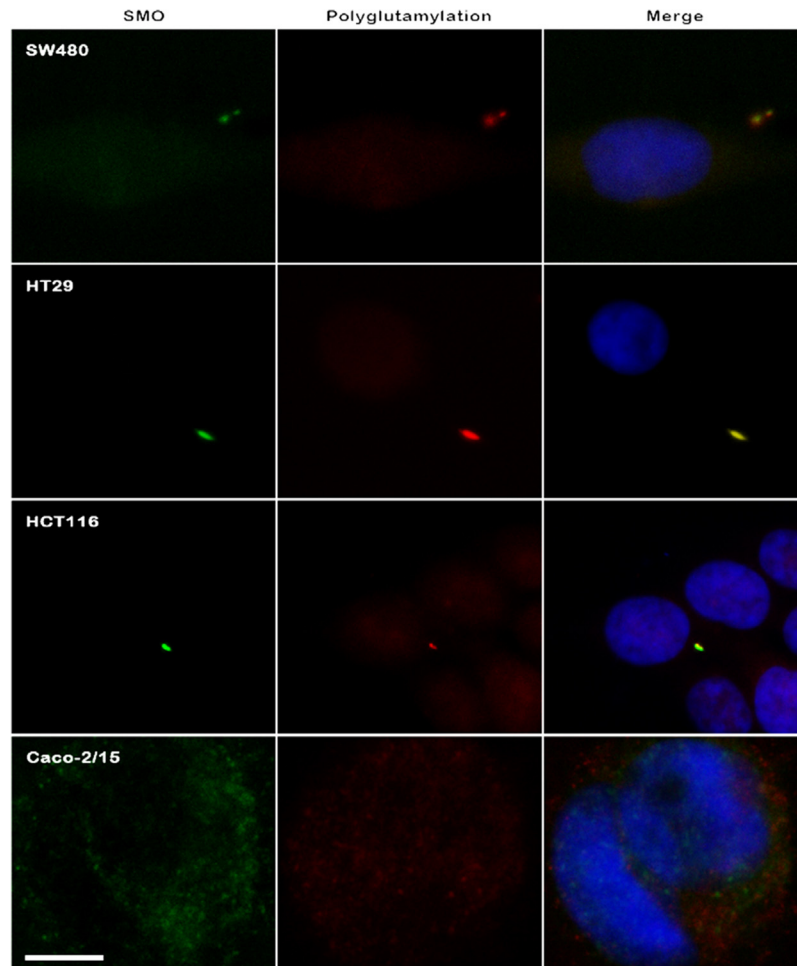


Fig. 6. Representative images of SMO localization in the PC detected by polyglutamylated tubulin in the CRC cell lines SW480, HT29, HCT116 and Caco-2/15. From the left. Column 1: SMO (green signal). Column 2: polyglutamylated tubulin detection of PC (red signal). Column 3: merge. Nuclei are stained blue DAPI. Scale bar: 50 μ m.

together, these results indicate a functional connection between PC and the HH pathway in colon cancer cells.

4. Discussion

To our knowledge, this is the first study reporting PC detection in colon cancer cells. The observation was unexpected considering that normal epithelial cells of the intestine and the colon have been specified to be PC negative based on the lack of cilia detection using typical cilia markers [2]. We confirmed herein the lack of PC detection in the normal intestinal epithelium. The possibility of PC in mouse normal epithelial colonic cells has been recently evoked on the basis of the detection of the glycolase TTLL3, a non-typical PC marker, while the use of antibodies against typical markers such as acetylated tubulin failed to specifically

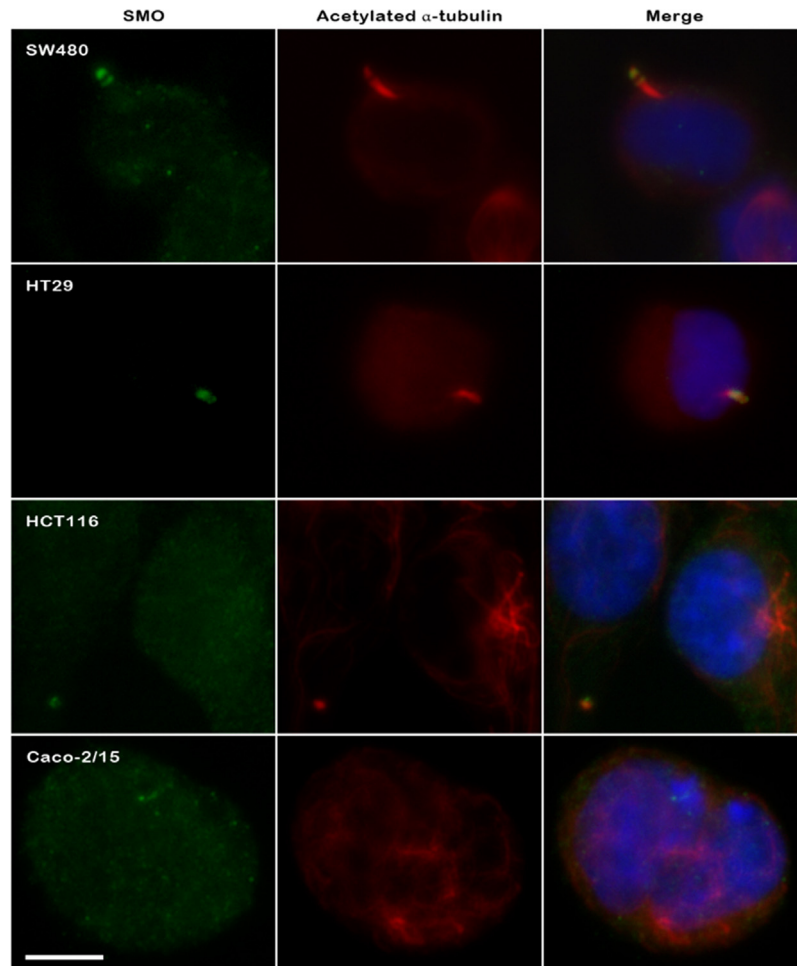


Fig. 7. Representative images depicting SMO localization in PC in the CRC cell lines HCT116, SW480, HT29 and Caco-2/15. Column 1: SMO (green signal). Column 2: acetylated α -tubulin detection of PC (red signal). Column 3: merge. Nuclei are stained blue with DAPI. Scale bar: 50 μ m.

detect PC [51]. The only site where we detected the PC in the normal colonic mucosa was the stroma as confirmed by double staining for the integrin $\alpha 6$ subunit, a specific epithelial cell marker [48]. For a long time PC were considered post-mitotic structures mainly related to the control of quiescence, making them irrelevant for tumor progression. Numerous observations have indeed been made in this direction. For instance, ciliogenesis appears to be impaired in many cancer types [31] including pancreatic cancer [52], cutaneous melanoma [53], glioblastoma and astrocytoma [54]. PC loss could participate in neoplastic transformation as shown in renal carcinoma, by disrupting cell polarity [55]. Not necessarily linked to altered proliferation, PC loss could be related to tubulin post-translational modification defects [4, 51], or mutations affecting proteins linking PC to the HH pathway such as Kif7 [56]. However, this point of view has been challenged over the past few years by the finding that PC-dependent pathways, especially the HH

pathway, are active in proliferating cells during development [14]. Furthermore, persistence of PC has been observed in other types of cancer, as in the gastrointestinal stromal tumor [57], medulloblastoma [30, 58], or in ovarian cancer, where PC may allow cells to escape from the cell cycle [59].

As for other cilia, the PC axoneme is characterized by post-translational modifications of tubulin, which are more or less specific to PC [4]. We have tested two of these with specific antibodies directed against acetylated α -tubulin and polyglutamylated tubulin, which are most often used to detect PC in various tissues [2, 60, 61]. Lysine 40 acetylation of the α -tubulin chain constitutes a ubiquitous modification, and is particularly strongly represented in PC [4]. Tubulin polyglutamylation consists of the addition of glutamate residues at the C terminus and is more specific to the PC and mitotic spindle, besides playing an active role in PC maintenance [62]. Using these antibodies, we have identified PC-like structures in 58% of CRC at all, including low (CRC I and II), stages but not in adenomas indicating that cilium appearance occurs relatively early in the carcinogenesis cascade but is not a feature associated with benign intestinal lesions.

It is noteworthy that the ubiquitous presence of the PC in stromal cells in both normal lamina propria and peritumoral stroma, as previously reported [2, 47], precluded any quantitative assessment of PC components in CRC tumors at the transcript or protein levels. In this context, the significant reduction of TLL3 observed in association with colon cancer progression [51] is difficult to interpret. We thus opted for another strategy based on the use of representative adenocarcinoma cell lines [40, 41, 43] to further investigate expression and functional aspects of the PC in CRC cells. The detection of PC in 3 of the 4 tested CRC cell lines was found to be consistent with the observation made in primary CRC tumors in which PC positive tumors accounted for ~60% of cases. Moreover, it is interesting to point out that the negative cell line, Caco-2/15, despite its tumoral origin, is often used as a “normal” model to study enterocytes, as they are able to spontaneously differentiate [42]. It is also worth mentioning that PC were not detected in all cancer cells of the PC-positive primary tumors and PC-positive CRC cell lines. Indeed, both in situ and in vitro, a significant proportion of CRC cells were found to be devoid of PC. This observation appears to be consistent with the fact that most cells disassemble their PC at cell cycle re-entry [11] while colon cancer cells cycle relatively rapidly. For instance under normal culture conditions, doubling times for the 4 colorectal cancer cell lines tested in the study are below 40 h (HT29: 21 h; HCT116: 23 h; Caco-2: 32 h; SW480: 38 h).

Analysis of the various components of the HH pathway in CRC cell lines showed that in addition to the two HH ligands SHH and IHH, known to be produced by normal intestinal epithelial cells [2, 47], expression of PC components PTCH, SMO and GLI1 was also observed in CRC cells in agreement with previous observations [32, 33, 34, 35, 36, 37], although a recent study suggests a loss of the

PC in colorectal adenocarcinoma cell lines, based on the relatively low expression of TTLL3 transcripts [51]. The expression of GLI1 is of particular interest since it is considered a key effector of the HH signaling pathway [37, 63]. In this context, it is worth noting that GLI1 was found to be expressed in the three PC-positive cell lines SW480, HT29 and HCT116, while minimally detected in the non-ciliated Caco-2/15 cells suggesting a functional relation between the presence of the PC and HH activity in colon cancer cells. To further investigate this possibility, expression of SMO on the surface of the PC was investigated. Indeed, in the canonical model of HH pathway activation, it is the recruitment of SMO to the PC membrane that triggers HH pathway activation [1, 19, 23, 24]. The identification of SMO at the surface of the PC in the three PC-positive and GLI1-expressing CRC cell lines SW480, HT29 and HCT116 as observed herein provided a strong indication that the PC and activity of the HH pathway are linked in CRC cells.

In conclusion, we thus propose that the PC can be considered as a marker of HH activity in CRC cells, and that regulation of the HH signaling pathway via the PC needs to be further explored in colorectal carcinogenesis.

Declarations

Author contribution statement

Blanche S enicourt: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Salah Boudjadi: Performed the experiments; Analyzed and interpreted the data.

Julie C. Carrier: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Jean-Fran ois Beaulieu: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Conflict of interest statement

The authors declare no conflict of interest.

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Additional information

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