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Hedgehog signaling pathway is inactive in colorectal cancer cell lines

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The Hedgehog (Hh) signaling pathway plays an important role in human development. Abnormal activation of this pathway has been observed in several types of human cancers, such as the upper gastro-intestinal tract cancers. However, activation of the Hh pathway in colorectal cancers is controversial. We analyzed the expression of the main key members of the Hh pathway in 7 colon cancer cell lines in order to discover whether the pathway is constitutively active in these cells. We estimated the expression of *SHH, IHH, PTCH, SMO, GLI1, GLI2, GLI3, SUFU* and *HHIP* genes by RT-PCR. Moreover, Hh ligand, Gli3 and Sufu protein levels were quantified by western blotting. None of the cell lines expressed the complete set of Hh pathway members. The ligands were absent from Colo320 and HCT116 cells, Smo from Colo205, HT29 and WiDr. *GLI1* gene was not expressed in SW480 cells nor were *GLI2*/*GLI3* in Colo205 or Caco-2 cells. Furthermore the repressive form of Gli3, characteristic of an inactive pathway, was detected in SW480 and Colo320 cells. Finally treatment of colon cancer cells with cyclopamine, a specific inhibitor of the Hh pathway, did not downregulate *PTCH* and *GLI1* genes expression in the colorectal cells, whereas it did so in PANC1 control cells. Taken together, these results indicate that the aberrant activation of the Hh signaling pathway is not common in colorectal cancer cell lines.

Key words: colon cancer; hedgehog; cyclopamine

The Hedgehog (Hh) signaling pathway is involved in the patterning of various tissues in many species.[1] The details of Hh pathway regulation are still being unravelled. Briefly, 3 vertebrate genes code for Hh ligands, Sonic- (*SHH*), Indian- (*IHH*), and Desert- (*DHH*) Hh. The ligands are processed and secreted and bind the Patched (Ptch) transmembrane receptor at the surface of responding cells. Ligand binding overcomes the repressive action of Ptch on the Smoothened (Smo) receptor and triggers the activation and nuclear translocation of Gli zinc finger transcription factors. Vertebrate cells contain 3 *GLI* genes, *GLI -1, -2* and *-3*. Gli2 and Gli3 proteins possess transcription activation and repression domains, while Gli1 has only an activation domain. In unstimulated cells Gli2 and Gli3 are cleaved to give rise to a repressive N-terminal form. Hh pathway activation inhibits Gli2 and Gli3 cleavage and stabilizes the full-length transcription factors that activate transcription.[2,3] It seems likely that Gli2 and Gli3 are the initial transducers of the Hh signal, necessary for the induction of *GLI1* expression.[4–7] Gli1 activates transcription of most of the Hh pathway target genes.[8–10] The transmembrane protein Hhip (Hh interacting protein) binds Hh ligands and attenuates the signal.[11,12] Recently, Sufu (suppressor of fused) was identified as the main repressor of the mammalian Hh signaling pathway.[13] Sufu inhibits the Hh signal by binding Gli factors in the cytoplasm and the nucleus.[14]

The *GLI1*, *PTCH* and *HHIP* genes are pathway members and target genes, their expression being upregulated when the signal is triggered.[11,15,16]

The Hh pathway is involved in gastro-intestinal tract development, as documented by the phenotype of mice where genes coding for Hh pathway members have been inactivated. Indeed, colon malformations have been observed in $Shh^{-/-}$, $Ihh^{-/-}$, $Gli2^{-/-}$ and $Gli3^{-/-}$ mice.[17] Organization in crypts has been shown to be lost in the colonic epithelium of $Ihh^{-/-}$ mice.[18,19] Moreover, in a rat model, Ihh has been shown to restrict the activity of the WNT/β-catenin pathway to cells located at the base of the crypts and thus maintain the differentiation of colonocytes at the tip of the crypts.[19]

The Hh pathway is constitutively activated in some types of human cancers.[20] Loss of function mutations of *PTCH* or *SUFU* genes, and gain of function mutations of *SMO* gene have been shown to be responsible for ligand independent activation of the pathway in basal cell carcinoma, medulloblastoma, or rhabdomyosarcoma. In most other tumor types the pathway has been shown to be activated by ligand overexpression.[21]

Several studies have aimed at understanding the role of the Hh pathway in colon cancer, but their results have been conflicting. Rare mutations of *PTCH* and *SMO* genes have been detected in this cancer type but they do not seem to affect the activity of the proteins.[22,23] The expression of a limited number of Hh pathway members, as well as the response of colon cancer cells to treatment with the Smo inhibitor cyclopamine, did not allow to lead to a conclusion concerning the activation of the pathway in this tumor type.[17,24]

Our first goal was thus to find out whether a thorough analysis of the expression of the members of the Hh pathway in colon cancer cells could help in coming to a conclusion on the ligand dependent activation of the pathway in colorectal cancer cell lines. So, we estimated the expression of Shh, Ihh, Ptch, Smo, Gli1, Gli2 and Gli3, which transmit the signal. We also analysed the expression of the negative regulators Sufu and Hhip.

Seven colon cancer cell lines were included in our study. None of our cell lines expressed all the key members of the Hh pathway. We then verified whether cyclopamine modulated the expression of *PTCH* and *GLI1* genes. Ptch and Gli1 transcripts levels were not significantly altered in cyclopamine treated colon cancer cells, in contrast with the cyclopamine responsive PANC cells. Our results thus suggest that Hh pathway activation is not a common event in colon cancer cell lines.

Material and methods

Cell culture

Colo205, Colo320, HT29 cell lines were purchased from American Type Culture Collection (Manassas, VA). WiDr, Caco-2, SW480, HCT116, and 293T cells were provided by Dr V. Bours (University of Liege, Belgium). PANC-1 cells were a gift from Dr. Kiss (University of Brussels, Belgium). Colo205, Colo320 cells were grown in RPMI1640, HT29 and HCT116 in McCoy'5A, WiDr in EMEM, SW480 in Leibovitz L-15, PANC1, and 293T in DMEM media. Culture media were supplemented with 10% FBS, 2 mM glutamine, 1 μg/ml penicillin/streptomycin.

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TABLE I – SEQUENCES OF THE PRIMERS USED IN THIS STUDY. THE PRIMERS WITHOUT A REFERENCE WERE DESIGNED IN THE LABORATORY

5 Gene name	Forward primer	Reverse primer	Reference
Ihh	CGGCTGACAATCACACGGAGC	GAAGCTGCCCTCTTCTAGCAG	
Shh	GACGACGGCGCCAAGAAGGT	ATGAGAATGGTGCCCTGGGC	
Ptch	CATCAACTGGAACGAGGACA	AGGGGCTTGTAAAACAGCAG	
Smo	CTGGTGTGGTTTGGTTTGTG	TGGTCTCGTTGATCTTGCTG	25
Gli1	CGGGGTCTCAAACTGCCCAGCTT	GGCTGGGTCACTGGCCCTC	
Gli2	CTAGCATCAGCGAGAACGTG	AAAGCCTAACTGGCATCCTC	
Gli3	CAGATGTGACGGAGAAAGCC	GATGATAGTATTCTGCTGGG	26
Sufu	CCAATCAACCCTCAGCGGCAGAATG	GTAGGTGAGAAAGAGGGCTGTC	
Hhip	AAAACAGATCATCAGCCAGAA	TAAATGCCCATTGGAATAGAAT	
Cph	TCTCCTTTGAGCTGTTTGCAGAC	AAACTTAACTCTGCAATCCAGC	

Caco-2 cells were grown in EMEM supplemented with 20% FBS, 2 mM glutamine, 1 μg/ml penicillin/streptomycin, 1 mM sodium pyruvate, 1.5 g/l sodium bicarbonate and 0.1 mM nonessential amino acids (Cambrex, Verviers, Belgium).

RNA extraction and semi-quantitative RT-PCR

Total RNA was extracted using the Tripure reagent (Roche) according to the manufacturer's instructions. To avoid genomic DNA contamination, RNA (20 μg) was treated with 20 U RNase-free DNaseI (Roche) at 37°C for 30 min, phenol–chloroform extracted and ethanol precipitated.

Semi-quantitative RT-PCR was performed in 2 steps. First, 2 μg of total RNA were reverse transcribed (RT) with 10 U of reverse transcriptase (AMV-RT, Promega, Madison WI), 0.8 μM specific primer, 0.4 mM dNTPs (Roche), 2 U RNase inhibitor (Promega) for 1 hr at 48°C. Next, PCR amplification was performed on 1/25th of the RT reaction with 2.5 U of AmpliTaq DNA polymerase (Applied Biosystems, Foster City, Ca), 0.2 μM of each primer, and 0.4 mM of dNTPs in a thermocycler (Perkin Elmer). Sequences of the primers used in this study are presented in Table I. Each RT-PCR product (10 μl) was analyzed by electrophoresis through a 1% agarose gel. PCR amplification signals were quantified by densitometric scanning using Quantity One (Bio-Rad).

Western blot analysis and antibodies

Cells cultured in 58 cm^2 Petri dishes were washed twice, collected in 1 ml of PBS and centrifuged. The cellular pellet was suspended in 400 μl of Buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT and 0.5 mM PMSF) and incubated 15 min on ice. Cytoplasmic and nuclear proteins were extracted from cells lysed with NP40 (1% final concentration). After centrifugation, the supernatant containing mainly cytoplasmic proteins was collected. Nuclei from the pellet were lysed with Buffer C (20 mM HEPES pH7.9, 0.5 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and 1 mM PMSF). Total proteins were extracted from cells lysed with 0.5% SDS. Protein concentrations were measured using the BCA protein assay method (Pierce, Rockford, IL). Either thirty micrograms of cytoplasmic and nuclear proteins or 50 μg of total cellular proteins were analysed by SDS-PAGE.

Secreted proteins were precipitated from conditioned media of 80% confluent cells grown in serum-free medium for 5 days. Proteins from 7 ml of conditioned media were acetone precipitated (final concentration 75%) and collected by centrifugation at 9000*g* for 15 min. The pellet was dissolved in 100 μl of SB buffer (SDS 0.33% (w/v), β-mercaptoethanol 0.83% (v/v), Tris 11.25 mM pH6.8, glycerol 1.66% (w/v) and bromophenol blue). Forty microliters were analysed by SDS-PAGE.

Proteins extracted from cells and conditioned media were separated on a 6% (Gli3) or 12% (Hh and Sufu) SDS-PAGE and transferred to PVDF membrane (Millipore, Billerica, MA). Membranes were blocked overnight in TBST buffer (Tris 50 mM pH 8.0, NaCl 150 mM, Tween20 0.1%) containing 5% low fat milk. They were then incubated for 2 hr with the following primary antibodies diluted in TBST buffer/5% low fat milk: 1/500 rabbit anti-Hh antibody (H-160, Santa Cruz Biotechnology, Santa Cruz, Ca), 1/500 rabbit anti-Gli3 (H-280, Santa Cruz), 1/500 goat anti-Sufu (C-15, Santa Cruz) 1/10,000 mouse anti-actin (MP biomedicals, Irvine, CA), 1/1,000 goat anti-Ku70 (Santa Cruz). Membranes were washed and incubated for 1 hr with the following secondary antibodies diluted in TBST buffer containing 5% milk: 1/2,000 goat anti-rabbit (Shh, Gli3), 1/2,000 goat anti-mouse and 1/1,000 (Sufu) or 1/4,000 (Ku70) rabbit anti-goat (DAKO, Carpinteria, CA). Signals were revealed with enhance chemiluminescence reagent (Amersham Biosciences, Little Chalfont, UK).

Transfection experiments

Gli3 cDNA was a kind gift from Dr B. Vogelstein. The Gli3 cDNA extracted with XbaI/SalI restriction enzymes was inserted in the XhoI/NheI sites of the pREP4 expression vector (Invitrogen, Paisley UK). Approximately 2×10^6 cells were seeded in 58 cm^2 Petri dishes 24 hr before transfection. PREP4-Gli3 expression vector (3 μg) was transfected with Fugene6 reagent (Roche) according to the manufacturer's recommendation. Nuclear proteins were extracted 24 hr later and analyzed as described above.

Cyclopamine and tomatidine treatment

Cyclopamine and tomatidine (Toronto Research Chemicals, North York, Ontario, CA) were dissolved in DMSO. About 1 to 2 $\times 10^6$ cells were seeded in 21 cm^2 Petri dishes in medium supplemented with 10% FBS. Twenty four hours later the medium was replaced by media containing 10 μM of cyclopamine, tomatidine or DMSO in 0.5% serum. After 24 hr the culture media were changed and the cells were harvested a further 24 hr later. Total RNA was extracted as described above. RT-PCR was performed as described above except that RT priming was performed with random hexamers (3 μg).

Results

The Hh pathway is not activated ligand dependently in colon cancer cells

Firstly, we analyzed the levels of the cellular and the secreted Hh protein by western blotting. Hh proteins are synthesized as 45 kDa precursors that are cleaved to give rise to the secreted 20 kDa factor. High levels of the 20 kDa processed Hh protein were detected in the cytoplasm (Fig. 1*a*) and the conditioned media (Fig. 1*b*) of HT29, WiDr and Caco-2 cells. Low levels of the ligand were detected in the cytoplasm and the conditioned media of Colo205 and SW480 cells (Figs. 1*a* and 1*b*). No Hh proteins were detected in Colo320 and HCT116 cells (Figs. 1*a* and 1*b*). The antibody does not discriminate between the different Hh ligands. To know which factor is expressed in the colon cancer cells we identified the Hh transcripts by specific RT-PCR. We focused our interest on Ihh and Shh, which seem to be the only Hh ligands expressed in the colon. Shh mRNA was present in all the cell lines, while Ihh transcripts were only detected in HT-29, WiDr and Caco-2 cells (Fig. 1*c*). Interestingly, the Ihh mRNA was present in cells expressing high protein levels.

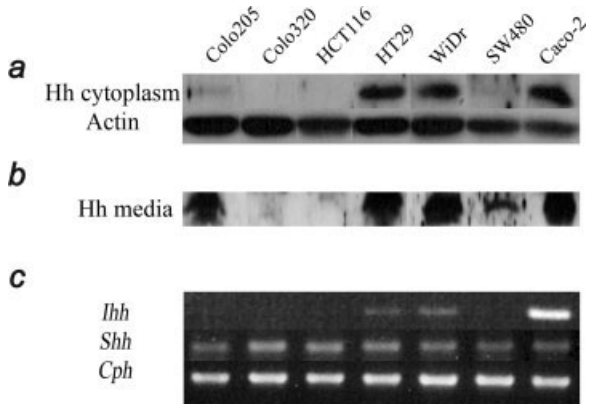

FIGURE 1 – Hh protein and mRNA levels in colorectal cancer cell lines. (*a*) Detection by western bloting of the 20 kDa mature Hh form in 30 μg of cytoplasmic protein extracts. Actin was used as loading control. (*b*) Detection of the mature Hh proteins in conditioned media with an antibody recognizing the 3 Hh forms. (*c*) Detection of Ihh and Shh transcripts by RT-PCR in the colon cancer cells using primer pairs that discriminate between these Hh transcripts. Cyclophilin (Cph) mRNA was amplified as control.

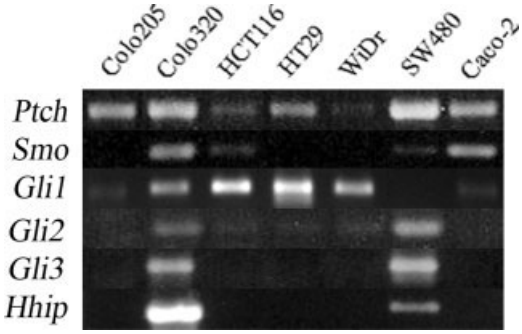

FIGURE 2 – Ptch, Smo, Gli1, Gli2, Gli3 and Hhip mRNA levels in the colorectal cancer cell lines. Two micrograms of total RNA were analysed by RT-PCR.

According to these results the Hh pathway could be activated through an autocrine mechanism in HT29, WiDr, Caco-2, Colo205 and SW480 cells.

To find out whether the colon carcinoma cells are able to respond to the Hh factors they produce, we evaluated the expression levels of Ptch and Smo receptors, and the 3 Gli transcription factors. Ptch transcripts were detected in every cell line, while Smo mRNA was present in Colo320, HCT116, SW480 and Caco-2 cells. The pattern of the expression of the 3 Gli transcription factors varied according to the cell line. Colo320 was the only cell line expressing all 3 Gli transcription factors. A strong signal corresponding to Gli1 mRNA and a weak Gli2 signal was detected in HCT116, HT29 and WiDr cells. Both Gli2 and Gli3 were present in SW480 cells, which in contrast did not express Gli1. Finally Caco2 and Colo205 cells expressed only very low levels of Gli1 (Fig. 2).

The Hh pathway regulates differentially the activity of the 3 Gli transcription factors. Hh stimulates *GLI1* gene transcription, while it inhibits the cleavage of Gli2 and Gli3 proteins. We analysed by western blotting the Gli3 protein present in our colorectal cancer cells. 293T cells transfected with a Gli3 expression vector served as positive control for this experiment. Nuclear proteins extracted from these cells were loaded on the same gel as the proteins extracted from the colon cancer cells, in order to distinguish the full length (Gli3FL) and the processed (Gli3R) Gli3. The Gli3 specific antibody revealed 3 bands of 190, 95 and 83 kDa in nuclear proteins from the parental 293T cells (Fig. 3). Only the intensity of the 190-kDa and the 83-kDa bands was greatly increased in Gli3 overexpressing 293T cells, indicating that these bands correspond respectively to Gli3FL and Gli3R. Both Gli3FL and Gli3R were detected in SW480 and Colo320 nuclear proteins, while Gli3 was not detected in the other cell lines, in agreement with the RT-PCR results.

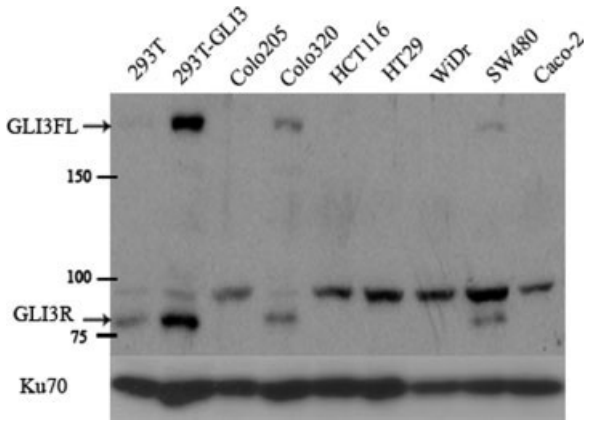

FIGURE 3 – Gli3 protein levels and cleavage status in colorectal cancer cells. Nuclear proteins (40 μg) extracted from the colorectal cancer cells, and from parental 293T cells and 293T cells transfected with a Gli3 expression vector (293T-Gli3) were analyzed by western blotting with an antibody recognizing the N-terminal part of the protein. The antibody reveals both the 190 kDa full-lengh protein (Gli3FL) and the 83 kDa cleaved repressor protein (Gli3R). Arrows indicate positions of Gli3FL and Gli3R proteins. Ku70 was used as a loading control.

Finally, we detected Hhip mRNA in Colo320 and SW480 cells, the only cells that also express the *GLI3* gene (Fig. 2).

These results thus suggest that no ligand dependent activation of the Hh pathway occurred in the colon cancer cells we study. Indeed, in the absence of ligands, the pathway cannot be activated in HCT116 and Colo320 cells. The absence of Smo does not allow the activation of the pathway in Colo205, HT29 and WiDr cells. The low level of Gli1 added to the absence of Gli2 and Gli3 in Caco2 cells and the absence of Gli1 from SW480 cells is a further indication that, in these cells, the pathway is probably also inactive. However, the Hh pathway could be activated downstream of the ligand, by mutations of Ptch, Smo or Sufu or by Smo activation through endogenous molecules. We tested the possibility of ligand independent activation of the Hh pathway in our colon cancer cell lines by Sufu mutation and by Smo activation.

The Hh pathway is not activated by ligand independent mechanisms in colon cancer cells

We first identified Sufu transcripts and protein in our colon cancer cells. Indeed, the absence of the protein or the production of truncated proteins due to *SUFU* gene mutations has been shown to be responsible for the pathway activation in some cancers.[22,23,27,28] We detected Sufu transcripts in the 7 colon cancer cell lines we analysed (Fig. 4). Moreover, the 54 kDa Sufu protein was present in 6 out of 7 cell lines. In Colo205 a shorter 50 kDa protein was detected. Next, we measured the modulation of the expression of Hh target genes in cyclopamine treated cells. Cyclopamine, an alkaloid from *Veratrum californicum*, binds and inactivates Smo and inhibits the pathway.[29] As positive control, we used PANC1 cyclopamine responsive pancreas cancer cells.[30] These cells express Shh, Ptch, Smo, Gli1, Gli2, Hhip and Sufu transcripts (Fig. 5*a*). As a negative control the cells were treated either with tomatidine, an alkaloid structurally close to cyclo-

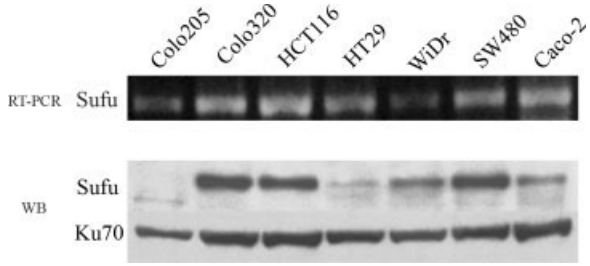

FIGURE 4 – Sufu mRNA and protein expression in colorectal cancer cell lines. Sufu transcripts were amplified by RT-PCR performed on 2 μg of total RNA. Sufu protein was detected in 50 μg of total protein extracts with an antibody recognizing the 54 kDa Sufu variant. Ku70 was used as a control.

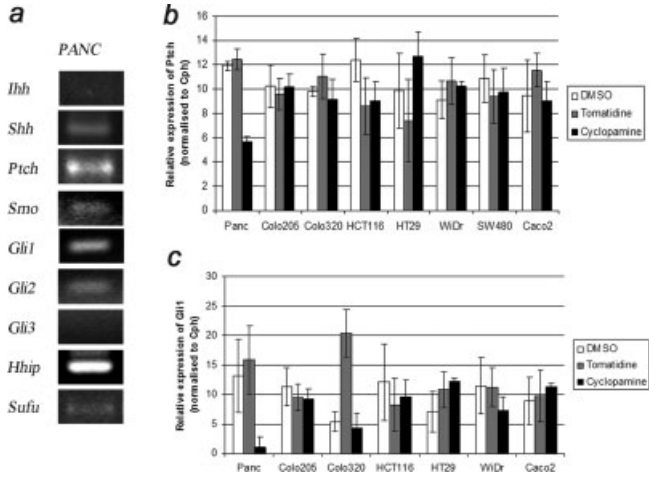

FIGURE 5 – Effect of cyclopamine on the expression of *PTCH* and *GLI1* genes in colorectal cancer cell lines. (*a*) Expression of Hedgehog pathway members in PANC cell line. (*b* and *c*) Ptch (*b*) and Gli1 (*c*) transcripts levels in response to DMSO, tomatidine (10 μM) or cyclopamine (10 μM) treatments. Expression of Ptch and Gli1 was analyzed by semi-quantitative RT-PCR. PCR signals were quantified by densitometry. Values were normalized to those of the Cph signal. Values represent mean ± S.D. of 3 independent experiments.

pamine but inactive on the Hh pathway, or with the solvent (DMSO).

Cyclopamine downregulated Ptch transcript levels in PANC cells, in comparison with the expression in tomatidine treated cells (Fig. 5*b*). In contrast, no significant modifications of the Ptch mRNA level were observed in the 7 colorectal cancer cells after cyclopamine treatement. Cyclopamine induced a sharp decrease in the Gli1 mRNA level in PANC cells (Fig. 5*c*). However, Gli1 mRNA levels were not significantly modified in cyclopamine treated colon cancer cells in comparison with control cells. Surprisingly, tomatidine upregulated Gli1 expression in Colo320 cells (Fig. 5*c*). Cyclopamine did not affect Hhip mRNA levels in any of the cells we tested, including the PANC cell line (data not shown).

Our results thus suggest that in the colon cancer cells we examined the Hh pathway is not activated by Sufu mutation or by aberrant activation of Smo.

Taken together, our results suggest that the constitutive activation of the Hh signalling pathway is not common in colon cancer cell lines.

Discussion

The published results on Hh pathway activation in colon cancer, based on the expression of some Hh target genes and on the response to cyclopamine treatment or the activity of a Gli responsive reporter vector, are conflicting. We wanted to discover whether a thorough analysis of the expression of the main key members of the Hh signaling pathway could help in understanding the activation status in colon cancer cell lines. Indeed, we think that it is important to define a reliable marker or combination of markers for Hh signal activity. This could then be applied in the identification of the human cancers that would benefit from a therapy targeting this pathway. To this end, we estimated the levels of Shh, Ihh, Ptch, Smo, Gli1, Gli2, Gli3, Sufu and Hhip transcripts and Hh, Gli3 and Sufu proteins in 7 cell lines derived from different grade colon cancers. None of the cell lines expressed all the key members required for the pathway's activation. Moreover, cyclopamine did not modulate the expression levels of *PTCH* and *GLI1* genes, further suggesting that the Hh pathway is not activated in the colon cancer cell lines we analyzed.

The cell lines we used have been tested in other studies for the expression of some Hh pathway members.[23,30,31] Concordant results were obtained for the expression of some pathway members and differences were noticed for others. In our opinion these discrepancies might be the consequences of epigenetic modifications to gene expression. Indeed, Ihh expression has been shown to be induced in HT29 cells by sodium butyrate, a HDAC inhibitor.[19] Moreover, Smo has been shown to be re-expressed upon treatment of colon cancer cells with a DNA methylation and a HDAC inhibitor.[23] Discrepancies were observed between our results and some published data for *PTCH* and *GLI1* genes expression in colon cancer cell lines. They are important since the expression of these genes is used as marker of Hh pathway activation. However, *PTCH* expression is not controlled only by the Hh signal. *PTCH* gene contains at least 3 alternative 5′ exons,[32] each with its own promoter, but only 1 contains a Gli binding site and responds to Hh pathway activation.[33] Moreover, Berman *et al.*[30] detected the Gli1 transcript in some colon cancer cells, although the transcription factor did not activate a Gli dependent reporter vector.

HHIP gene expression is increased in Hh stimulated cells. *HHIP* was expressed in 2 of our cell lines, Colo205 and SW480. However, the case of Hhip is complex, because besides being a target gene it is a powerful inhibitor of the pathway as shown in transgenic mice[34] and in cell lines.[12] Hhip downregulation has been shown to increase the Hh signal in some pancreatic cancer cell lines.[35] *HHIP* expression has been shown to be reduced in 8/10 primary human colon cancers, when compared with the normal tissue from the same patient.[12] The contribution of Hhip to the inhibition of the Hh pathway in colon cancer cells will have to be assessed experimentally.

The presence of the full-length Gli2 and Gli3 proteins, but not the transcript or total protein levels indicates the activation of the pathway. The absence of Gli2 probably impairs the transmission of the signal in Caco2 and Colo205 cells. We cannot propose a role for Gli2 in cells where the transcript was present since we could not examine the cleavage status of the protein. In contrast, we were able to show the presence of the repressive Gli3R in 2 cell lines. To our knowledge this is the first report of the presence of this repressor in colon cancer cells. Interestingly, mice engineered to express a C-terminally truncated Gli3 protein have been shown to present gastro-intestinal abnormalities similar to those of $Shh^{-/-}$ or $Ihh^{-/-}$ animals.[36] The role of these transcription factors in colon cancer should be studied further.

Our expression results do not rule out the possibility of ligand independent activation of the Hh pathway in some cell lines. We examined the possibility of ligand independent aberrant activation of the Hh pathway due to the absence of *SUFU* or the expression of truncated SUFU protein as well as by constitutive Smo activation.

Sufu transcripts were present in every colon cancer cell line we tested. More importantly, the full-length protein was present in 6

of the 7 cell lines. The mechanism responsible for the shorter protein production in Colo205 cells will have to be determined. Sufu has been shown to inhibit the activity of all 3 Gli transcription factors. Sufu mutations lead to increased Gli activity and target genes expression in different cancer types.[28,37] Although most mutations observed in cancer cells give rise to shorter proteins, we cannot rule out the presence mutations which would modify the activity of the protein. Further work is thus needed to ascertain the role of Sufu in colon cancer cell lines.

Smo can be activated independently of Hh by mutation and by endogenous metabolites.[38] Cyclopamine inhibits Smo activated by these mechanisms. We did not detect significant differences in the expression of the *PTCH* and *GLI1* genes between cyclopamine and tomatidine treated colon cancer cells. In contrast, both Ptch and Gli1 transcript levels were downregulated in cyclopamine but not in tomatidine treated PANC cells used as positive control. Our results agree with those of Berman *et al*. who did not observe a modulation of *PTCH* expression in cyclopamine treated HCT116 cells, and the drug did not increase the apoptosis of these cells in comparison with tomatidine.[30] In contrast, cyclopamine increases apoptosis rate of colon cancer cells according to Qualtrough *et al*.[31] However, these authors did not use tomatidine to control for possible non specific effects of cyclopamine.

Cell lines might not be good models for the assessment of Hh pathway activity.[21] However, the published results on primary human colon cancers are also confusing. Some authors,[39,40] but not others[22] detected increased levels of Hh pathway members during colon cancer progression. Moreover, the expression of Ihh and Gli1 were shown to be decreased during colon cancer progression in recent publications.[19,41]

Hh pathway activity might be involved in the progression of colon cancer *in vivo* by mediating the cross-talk between the cancerous epithelial cells and the cells from the tumor environment. Also, we cannot exclude that the Hh pathway is activated in a cellular subpopulation, for instance in cancer stem cells. It would be interesting to re-examine this in the recently identified colon cancer stem cells.[42,43]

Our results and the data from the literature rise the question of what is a reliable marker of Hh pathway status in cells were the pathway is not manipulated. Identification of post-transcriptional modifications, such as processing, sub-cellular localization or phosphorylation of some Hh pathway members might predict more accurately the Hh pathway activity than expression levels. Clearly more work is needed to understand the possible involvement of this pathway in colon cancer before it can be considered as a suitable therapeutic target.

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