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SMO Expression in Colorectal Cancer: Associations with Clinical, Pathological and Molecular Features

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Abstract

Background—SMO (the official symbol for "smoothened, frizzled family receptor") is an important component of the hedgehog signaling pathway, which has been implicated in various human carcinomas. However, clinical, molecular, and prognostic associations of SMO expression in colorectal cancer remain unclear.

Methods—Using a database of 735 colon and rectal cancers in the Nurse's Health Study and the Health Professionals Follow-up Study, we examined the relationship of tumor SMO expression (assessed by immunohistochemistry) to prognosis, and to clinical, pathological and tumor molecular features, including mutations of *KRAS*, *BRAF* and *PIK3CA*, microsatellite instability,

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Conflict of Interest Statement:
No potential conflicts of interest exist.

Use of standardised official symbols: We use HUGO (Human Genome Organisation)-approved official symbols for genes and gene products, including BRAF; SMO (smoothened, frizzled family receptor); PIK3CA, AKT and KRAS; all of which are described at www.genenames.org.

CpG island methylator phenotype (CIMP), LINE-1 methylation, and expression of phosphorylated AKT and CTNNB1.

Results—SMO expression was detected in 370 (50%) tumors. In multivariate logistic regression analysis, SMO expression was independently inversely associated with phosphorylated AKT expression [odds ratio (OR), 0.48; 95% confidence interval (CI), 0.34–0.67] and CTNNB1 nuclear localization (OR, 0.48; 95% CI, 0.35–0.67). SMO expression was not significantly associated with colorectal cancer-specific or overall survival. However, in CIMP-high tumors, but not CIMP-low/0 tumors, SMO expression was significantly associated with better colorectal cancer-specific survival (log-rank $P = 0.012$; multivariate hazard ratio, 0.36; 95% CI, 0.13–0.95; $P_{interaction} = 0.035$, for SMO and CIMP status).

Conclusions—Our data reveal novel potential associations between the hedgehog, the WNT/CTNNB1, and the PI3K (phosphatidylinositol-4,5-bisphosphonate 3-kinase)/AKT pathways, supporting pivotal roles of SMO and hedgehog signaling in pathway networking. SMO expression in colorectal cancer may interact with tumor CIMP status to affect patient prognosis, although confirmation by future studies is needed.

Keywords

colon carcinoma; rectal cancer; hedgehog; HH; molecular pathology; clinical outcome

INTRODUCTION

Colorectal cancers represent a heterogeneous group of complex multifactorial diseases, which are influenced by host and environmental factors.[1] Molecular classification (e.g. by *KRAS*, *BRAF*, and MSI status) has become essential in both research and clinical practice to better predict tumor progression and behavior.[2–5]

The hedgehog signaling pathway plays a role in patterning, growth, and differentiation in various tissues, including the gastrointestinal tract.[6–8] In mammals, hedgehog signaling is initiated through binding of one of three ligands [sonic hedgehog (SHH), indian hedgehog (IHH) and desert hedgehog (DHH)] to the trans-membrane receptor patched 1 (PTCH1), leading to release of the suppressed transmembrane protein smoothened, fizzled family receptor (SMO) and subsequent activation of GLI transcription factors.[6] Hedgehog signaling has been implicated in the pathogenesis of various human cancers, either through hedgehog ligand-dependent activation, or through ligand-independent activation, i.e., by loss of function mutations in *PTCH1*, or gain of function mutations in the proto-oncogene *SMO*.[9–11] Consequently, the hedgehog pathway is viewed as a potential therapeutic target.[9,12]

Although evidence supporting a role of the hedgehog pathway in colorectal neoplasia has tended to be inconsistent,[13–21] accumulating experimental data demonstrate that the hedgehog signaling pathway cooperates with other molecular alternations and signaling pathways, such as WNT signaling,[22,23] and phosphatidylinositol-4,5-biphosphonate 3-kinase (PI3K) /AKT,[24–26] in multiple tumorigenic contexts, even in the absence of hedgehog ligand-dependent pathway activation.[22,25]

Given evidence of cross-talk between hedgehog and other signaling pathways in human carcinogenesis, we hypothesized that SMO expression in colorectal cancer might be associated with other important tumor characteristics, such as CTNNB1 and phosphorylated AKT expression. We therefore utilized a molecular pathological epidemiology database,[27,28] derived from colorectal cancers arising in two U.S. nationwide prospective cohort studies, to examine SMO expression status in colorectal cancer, and to assess the relationships between SMO expression and other important molecular features, including: microsatellite instability (MSI); CpG island methylation phenotype (CIMP); long interspersed nucleotide element-1 (LINE-1) methylation; and *KRAS*, *BRAF*, and *PIK3CA* mutations. We also sought to evaluate the prognostic association of SMO expression, and to explore the potential for its interaction with other tumor features in survival analyses.

MATERIALS AND METHODS

Study Group

We used the database of two prospective cohort studies, the Nurses' Health Study (NHS, N = 121,700 women observed since 1976) and the Health Professionals Follow-Up Study (HPFS, N = 51,500 men observed since 1986).[29] Participants were sent follow-up biennial questionnaires to update information on diet and lifestyle factors, and to identify newly diagnosed cancers and other diseases. In this population-based study, besides medications that a given patient took by themselves, treatment modality was chosen by treating physicians, and detailed treatment data were not available. After confirmation of colorectal cancer, we requested paraffin embedded tissue blocks from hospitals across the U.S., where participants had undergone resection of primary tumors. We were able to obtain colorectal cancer specimens for 1443 cases out of 3019 colorectal cancer cases recorded up to June 2006. Diagnostic biopsy specimens from rectal cancer patients who received pre-operative therapy were collected in order to avoid treatment-related artifact. Tumor location was categorized [cecum; ascending colon (including hepatic flexure); transverse colon; descending colon (including splenic flexure); sigmoid colon; rectum] based on medical records.[30] All colorectal cancer cases were confirmed through review of histology by a pathologist (S.O.) blinded to exposure data. Tumor grade was categorized as high (≤50% glandular area) or low (> 50% glandular area). Based on the availability of SMO expression data and survival data, a total of 735 colorectal cancer cases diagnosed up to 2006 were included in this study. Patients were observed until death, or January 2011, whichever came first. Death of a participant was ascertained through the National Death Index, or by reporting by family members or postal authorities. The cause of death was assigned by study physicians. Written informed consent was obtained from all study subjects. Human Subjects Committees at Harvard School of Public Health and Brigham and Women's Hospital approved this study.

Immunohistochemistry for SMO, Phosphorylated AKT and CTNNB1

Tissue microarray blocks were constructed as previously described.[31] Methods of immunohistochemical staining and interpretations for phosphorylated AKT (at amino acid position Ser 473) and CTNNB1 have been described previously.[32–34] For SMO immunostaining, deparaffinized tissue sections were heated in a microwave for 15 minutes

in Antigen Retrieval Citra Solution, pH 6 (BioGenex Laboratories, San Ramon, CA). Tissue sections were incubated with Dual Endogenous Enzyme Block (DAKO, Carpinteria, CA), then Serum Free Protein Block (DAKO), each for 15 minutes. Slides were incubated at room temperature for one hour with a primary antibody against SMO (1:100, rabbit polyclonal; Santa Cruz, sc-13943, San Diego, CA). Envision™ anti-rabbit HRP-labeled polymer (DAKO) was applied to the sections for 30 minutes, followed by visualization using the chromogen 3,3-diaminobenzidine (DAKO), and hematoxylin counterstain. The specificity of the SMO antibody was confirmed by previous studies in different tissues and cells.[35–37] Positive and negative controls were included in each panel of immunohistochemistry for all markers.[33,34] Known positive prostate carcinoma was used as a positive control for SMO.[38] Sections processed with replacement of primary antibody by Tris-buffered saline were used as a negative control.

For each case, cytoplasmic SMO status was recorded as absent, weak, moderate or intense staining. SMO expression was defined as the presence of weak to intense staining (Figure 1). Immunostained tissue for each marker was scored by a single pathologist (SMO by X.L.; phosphorylated AKT by Y.B.; and CTNNB1 by T.M.) blinded to other data. A subset sample of over 100 cases for each marker was scored independently by a second pathologist (SMO by T.M.; phosphorylated AKT by K.S.; and CTNNB1 by S.O.) unaware of other data. The concordance between the two observers (all $P < 0.0001$) was 0.91 ($\kappa = 0.79$, N = 118) for SMO, 0.81 ($\kappa = 0.59$, N = 132) for phosphorylated AKT, and 0.90 ($\kappa = 0.80$, N = 292) for nuclear CTNNB1 localization, indicating good to substantial agreement.

Sequencing of *KRAS*, *BRAF* and *PIK3CA* Mutation, and Analysis for Microsatellite Instability

Genomic DNA was extracted from paraffin-embedded tissue. PCR and pyrosequencing targeted at *KRAS* [codons 12 and 13 (since 90% of *KRAS* mutations occur in these two codons)],[32,39] *BRAF* (codon 600),[40,41] and *PIK3CA* exons 9 and 20,[42] were performed as previously described. Microsatellite instability was assessed using a panel of 10 microsatellite markers (D2S123, D5S346, D17S250, BAT25, BAT26, BAT40, D18S55, D18S56, D18S67 and D18S487).[41] MSI-high was defined as the presence of instability in 30% or more of the markers, and MSI-low/microsatellite stability (MSS) as instability 0–29% of the markers.[41]

Real-time PCR for CpG Island Methylation and Pyrosequencing to Measure LINE-1 Methylation

Sodium bisulfite treatment of DNA, and real-time PCR assays (MethyLight) were performed as previously described.[41,43,44] We quantified promoter methylation at eight CIMP-specific loci: *CACNA1G, CDKN2A* (p16), *CRABP1, IGF2, MLH1, NEUROG1, RUNX3,* and *SOCS1*. CIMP-high was defined as ≥ 6 (of 8) methylated promoters, and CIMP-low/0 as 0–5 (of 8) methylated promoters. To accurately quantify methylation level in LINE-1, a PCR-pyrosequencing assay was employed.[45,46]

Statistical Analysis

All statistical analyses were performed using SAS software (version 9.2; SAS Institute Inc, Cary, NC). All *P* values were two-sided. When multiple hypothesis testing was performed, the *P* value for significance was adjusted to $P = 0.0033$ (= 0.05/15) by Bonferroni correction. For categorical data, the chi-square test or Fisher's exact test was performed. To compare mean age and mean LINE-1 methylation levels, a t-test, assuming equal variances, was performed.

The Kaplan-Meier method and the log-rank test were performed for survival analyses. Deaths from causes other than colorectal cancer were censored in colorectal cancer-specific mortality analyses. To control for confounding, we used Cox proportional hazards models to calculate hazard ratio (HR) of death according to tumor SMO expression status. The model initially included age at diagnosis (continuous), sex, year of diagnosis (continuous), body mass index, tumor location (proximal vs. distal colon vs. rectum), tumor grade, MSI (high vs. low/MSS), CIMP (high vs. low/0), LINE-1 methylation (continuous), *BRAF* mutation, *KRAS* mutation, and *PIK3CA* mutation, in addition to CTNNB1 and phosphorylated AKT expression. To minimize residual confounding and overfitting, disease stage (I, II, III, IV, or unknown) was used as a stratifying variable using the "strata" option in the SAS "proc phreg" command. To avoid overfitting, variables in the final model were selected using backward stepwise elimination with a threshold of $P = 0.05$. Interaction was assessed using the Wald test on the cross-product of SMO and another variable of interest (excluding cases missing data) in a multivariate Cox model. To improve efficiency of the models, cases with missing data in any of the categorical variables [CIMP (1.8%), MSI (2.0%), *BRAF* (1.3%), *KRAS* (1.1%), *PIK3CA* (9.8%), CTNNB1 (4.7%) and phosphorylated AKT (6.9%)], were included in the majority category for that variable. We confirmed that excluding cases with missing information in any of the covariates did not substantially alter the results (data not shown).

To assess whether associations between SMO expression and the variables in Table 1 were independent of other variables, a multivariate logistic regression analysis was conducted for cross sectional analyses. To calculate adjusted odds ratios (OR), the model initially included variables as in Cox proportional hazards models. To avoid overfitting, a backward stepwise elimination with a threshold of $P = 0.05$ was used to select variables in the final model. After the variables in the final logistic regression model were selected, we employed a missing indicator method for those cases with missing data in a given variable, to obtain a more accurate effect estimate in the given variable.

RESULTS

SMO Expression in Colorectal Cancer

Among 735 colorectal cancer cases diagnosed up to 2006 with SMO expression data, we observed SMO expression in 370 tumors (50%) by immunohistochemistry (Figure 1). SMO expression was positively associated with *KRAS* mutation ($P = 0.0027$), and inversely associated with phosphorylated AKT expression ($P < 0.0001$), *BRAF* mutation ($P = 0.0026$), CTNNB1 nuclear localization ($P = 0.0005$) and CIMP-high status ($P = 0.0035$) (Table 1).

Multivariate Logistic Regression Analysis to Assess Associations with SMO Expression in Colorectal Cancer

Multivariate logistic regression analysis was performed to assess independent relationships between SMO expression and other factors. Phosphorylated AKT expression [multivariate OR = 0.48; 95% confidence interval (CI), 0.34–0.67; $P < 0.0001$] and CTNNB1 nuclear localization (OR = 0.48; 95% CI, 0.35–0.67; $P < 0.0001$) remained significantly associated with SMO expression in the final model.

In addition, *BRAF*-mutation/*KRAS*-wild-type (vs. *BRAF*-wild-type/*KRAS*-wild-type) and CIMP-high remained in the final model [(OR = 0.49; 95% CI, 0.28–0.85; $P = 0.011$) and (OR = 0.59; 95% CI, 0.35–0.98; $P = 0.043$), respectively], but these associations were not statistically significant given multiple hypothesis testing (Table 2).

SMO Expression and Patient Survival in Colorectal Cancer

During follow-up of 735 patients with survival data (median follow-up time 14.1 years for censored cases), there were 373 deaths, including 216 deaths due to colorectal cancer. In Kaplan-Meier analyses, SMO expression was not significantly associated with colorectal cancer-specific survival (log-rank $P = 0.85$) or overall survival (log-rank $P = 0.72$).

We performed Cox proportional hazards regression models to assess mortality according to SMO status, but did not observe a significant association between SMO expression and survival in univariate, stage-stratified, or multivariate stage-stratified analyses (data not shown).

Interactions between SMO Expression and other Variables in Colorectal Cancer Survival Analysis

We examined whether any clinical, pathological, or molecular variables significantly modified the association of SMO expression with patient survival. We observed a borderline significant interaction between SMO expression and CIMP status in colorectal cancer-specific survival ($P_{interaction} = 0.035$, given multiple testing significance level was adjusted to $P = 0.0033$). For patients with CIMP-high tumor, SMO positivity was significantly associated with better colorectal cancer-specific survival (multivariate HR, 0.36, 95% CI, 0.13–0.95); whereas, for patients with CIMP-low/0 tumor, SMO positivity was not significantly associated with colorectal cancer-specific survival (Table 3). The differential effect of SMO expression on colorectal cancer-specific survival according to CIMP status was also evident in Kaplan-Meier analyses (Figure 2).

The association of SMO expression with cancer-specific mortality did not significantly differ according to any of the other variables.

DISCUSSION

In this study, the unique resource of a molecular pathological epidemiology database,[27,28] containing a large number of colorectal cancers, and prospectively collected data from two cohort studies, enabled us to comprehensively evaluate the associations of SMO expression with clinical, pathological and tumor molecular features. We observed that SMO was

expressed in around one half of colorectal cancers. In a multivariate logistic regression model, SMO expression was significantly inversely associated with phosphorylated AKT expression and CTNNB1 nuclear localization. An inverse association was also observed between SMO expression and *BRAF*-mutant/*KRAS* wild-type; however, the association was of borderline significance, when multiple testing was taken into account.

Recent studies have demonstrated that colorectal cancers constitute a group of heterogeneous tumors at the molecular level.[47,48] The development and progression of colorectal neoplasia is attributable to the accumulation of genetic and epigenetic changes and the complex interaction of aberrations in various signaling pathways.[49–52] Each tumor has its own unique characteristics in terms of molecular phenotype, tumor microenvironment, and interactomes within and between neoplastic and host cells.[1,53] Therefore, tumor biomarker testing contributes to personalized medicine research and, ultimately, to clinical practice.[50,54–56]

Experimental data suggest a link between SMO expression and the PI3K/AKT pathway. AKT is a major downstream effector of PI3K, and plays a crucial role in regulating a wide variety of cellular process, including cellular metabolism as well as cell proliferation and survival.[57] Riobo et al. have previously shown that PI3K and AKT are essential for SHH signaling.[24] Furthermore, SMO activity is required for cooperation between SHH and insulin-like growth factor in promoting myogenic proliferation and differentiation via the MAPK/ERK and PI3K/AKT pathways.[25] In our dataset, SMO expression was inversely associated with phosphorylated AKT expression in colorectal cancer, suggesting that SMO activation may tend to be mutually exclusive with AKT activation in colorectal cancer development.

Crosstalk between the hedgehog and WNT signaling pathways in intestinal tumorigenesis remains controversial.[7,20,22,50,58] Several groups have reported possible negative regulation of the WNT pathway by hedgehog signaling.[7,58] In one study, overexpression of IHH resulted in down-regulation of intestinal CTNNB1.[58] Nuclear expression of CTNNB1 has been found to be inversely associated with GLI1 staining in colorectal cancers, suggesting that GLI1 plays an inhibitory role in the development of colorectal cancer driven by WNT signaling.[20] However, Arimura et al. have shown that reduced SMO expression inhibits WNT signaling by down-regulating nuclear CTNNB1 expression, independent of GLI-mediated hedgehog signaling.[22] Our current findings suggest that tumors with SMO expression are inversely associated with CTNNB1 nuclear expression, favoring a negative regulation of the WNT pathway by hedgehog signaling.

While SMO expression was not associated with colorectal cancer-specific survival or overall survival, our data suggest a possible interaction with CIMP status in patient prognosis. CIMP constitutes an epigenomic phenomenon characterized by widespread promoter methylation, which leads to tumor suppressor gene silencing.[59] CIMP status has been extensively investigated in colorectal cancer.[60–65] In our current study, we observed that SMO expression was inversely associated with CIMP-high status. Moreover, our data suggest that, within CIMP-high cancers (but not within CIMP-low/0 cancers), patients with SMO-expressing tumors may expect better cancer-specific survival compared to those with

SMO-nonexpression tumors. Given multiple hypotheses testing, and the exploratory nature of our interaction analyses, these findings need confirmation by additional independent studies.

Interestingly, we observed a possible inverse association between SMO expression and *BRAF*-mutation/*KRAS*-wild-type in colorectal cancers, independent of other molecular variables. *BRAF* mutation is present in 10–15% of colorectal cancers and associated with inferior prognosis.[66–69] Nonetheless, our results need to be confirmed by independent studies.

There are also limitations in the present study. Firstly, data on treatment were limited. We speculated that chemotherapy administration did not substantially differ by tumor SMO expression, since the data were not available for treating physicians. Nevertheless, our regression analyses were adjusted for TNM stage, on which treatment decisions are largely based. Secondly, despite quite high agreement of readings of the two pathologists for SMO immunohistochemistry there was still a 9% discordance rate.

In conclusion, our large cohort study has shown that SMO expression in colorectal cancer is inversely associated with phosphorylated AKT expression and CTNNB1 nuclear localization. SMO expression in colorectal cancer may interact with tumor CIMP status to affect patient prognosis, although confirmation by future studies is needed. Our data are compatible with literature supporting a role for SMO in pathway networking in colorectal carcinogenesis.

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Abbreviations

CI	confidence interval
CIMP	CpG island methylator phenotype
HPFS	the Health Professionals Follow-Up Study
HR	hazard ratio

MSI	microsatellite instability
MSS	microsatellite stable
NHS	the Nurses' Health Study
OR	odds ratio
PI3K	phosphatidylinositol-4,5-biphosphonate 3-kinase
SD	standard deviation

References

1. Ogino S, Fuchs CS, Giovannucci E. How many molecular subtypes? Implications of the unique tumor principle in personalized medicine. Expert Rev Mol Diagn. 2012; 12:621–628. [PubMed: 22845482]
2. Funkhouser WK Jr, Lubin IM, Monzon FA, et al. Relevance, pathogenesis, and testing algorithm for mismatch repair-defective colorectal carcinomas: a report of the association for molecular pathology. J Mol Diagn. 2012; 14:91–103. [PubMed: 22260991]
3. Colussi D, Brandi G, Bazzoli F, Ricciardiello L. Molecular pathways involved in colorectal cancer: implications for disease behavior and prevention. International journal of molecular sciences. 2013; 14:16365–16385. [PubMed: 23965959]
4. Bardhan K, Liu K. Epigenetics and colorectal cancer pathogenesis. Cancers. 2013; 5:676–713. [PubMed: 24216997]
5. Kim JH, Kang GH. Molecular and prognostic heterogeneity of microsatellite-unstable colorectal cancer. World journal of gastroenterology : WJG. 2014; 20:4230–4243. [PubMed: 24764661]
6. Berman DM, Karhadkar SS, Maitra A, et al. Widespread requirement for Hedgehog ligand stimulation in growth of digestive tract tumours. Nature. 2003; 425:846–851. [PubMed: 14520411]
7. Kolterud A, Grosse AS, Zacharias WJ, et al. Paracrine Hedgehog signaling in stomach and intestine: new roles for hedgehog in gastrointestinal patterning. Gastroenterology. 2009; 137:618–628. [PubMed: 19445942]
8. Fendrich V, Oh E, Bang S, et al. Ectopic overexpression of Sonic Hedgehog (Shh) induces stromal expansion and metaplasia in the adult murine pancreas. Neoplasia. 2011; 13:923–930. [PubMed: 22028618]
9. Ng JM, Curran T. The Hedgehog's tale: developing strategies for targeting cancer. Nat Rev Cancer. 2011; 11:493–501. [PubMed: 21614026]
10. Teglund S, Toftgard R. Hedgehog beyond medulloblastoma and basal cell carcinoma. Biochim Biophys Acta. 2010; 1805:181–208. [PubMed: 20085802]
11. Michael LE, Westerman BA, Ermilov AN, et al. Bmi1 is required for Hedgehog pathway-driven medulloblastoma expansion. Neoplasia. 2008; 10:1343–1349. 1345–1349. [PubMed: 19048113]
12. Zhang Y, Laterra J, Pomper MG. Hedgehog pathway inhibitor HhAntag691 is a potent inhibitor of ABCG2/BCRP and ABCB1/Pgp. Neoplasia. 2009; 11:96–101. [PubMed: 19107236]
13. Chatel G, Ganeff C, Boussif N, et al. Hedgehog signaling pathway is inactive in colorectal cancer cell lines. Int J Cancer. 2007; 121:2622–2627. [PubMed: 17683069]
14. Oniscu A, James RM, Morris RG, Bader S, Malcomson RD, Harrison DJ. Expression of Sonic hedgehog pathway genes is altered in colonic neoplasia. J Pathol. 2004; 203:909–917. [PubMed: 15258993]
15. Qualtrough D, Buda A, Gaffield W, Williams AC, Paraskeva C. Hedgehog signalling in colorectal tumour cells: induction of apoptosis with cyclopamine treatment. Int J Cancer. 2004; 110:831–837. [PubMed: 15170664]
16. Alinger B, Kiesslich T, Datz C, et al. Hedgehog signaling is involved in differentiation of normal colonic tissue rather than in tumor proliferation. Virchows Arch. 2009; 454:369–379. [PubMed: 19280222]

17. Mazumdar T, DeVecchio J, Shi T, Jones J, Agyeman A, Houghton JA. Hedgehog signaling drives cellular survival in human colon carcinoma cells. Cancer Res. 2011; 71:1092–1102. [PubMed: 21135115]
18. Varnat F, Duquet A, Malerba M, et al. Human colon cancer epithelial cells harbour active HEDGEHOG-GLI signalling that is essential for tumour growth, recurrence, metastasis and stem cell survival and expansion. EMBO Mol Med. 2009; 1:338–351. [PubMed: 20049737]
19. Saqui-Salces M, Merchant JL. Hedgehog signaling and gastrointestinal cancer. Biochim Biophys Acta. 2010; 1803:786–795. [PubMed: 20307590]
20. Akiyoshi T, Nakamura M, Koga K, et al. Gli1, downregulated in colorectal cancers, inhibits proliferation of colon cancer cells involving Wnt signalling activation. Gut. 2006; 55:991–999. [PubMed: 16299030]
21. Ding YL, Wang QS, Zhao WM, Xiang L. Expression of smoothened protein in colon cancer and its prognostic value for postoperative liver metastasis. Asian Pac J Cancer Prev. 2012; 13:4001–4005. [PubMed: 23098507]
22. Arimura S, Matsunaga A, Kitamura T, Aoki K, Aoki M, Taketo MM. Reduced level of smoothened suppresses intestinal tumorigenesis by down-regulation of Wnt signaling. Gastroenterology. 2009; 137:629–638. [PubMed: 19427313]
23. Morris, JPt; Wang, SC.; Hebrok, M. KRAS, Hedgehog, Wnt and the twisted developmental biology of pancreatic ductal adenocarcinoma. Nat Rev Cancer. 2010; 10:683–695. [PubMed: 20814421]
24. Riobo NA, Lu K, Ai X, Haines GM, Emerson CP Jr. Phosphoinositide 3-kinase and Akt are essential for Sonic Hedgehog signaling. Proc Natl Acad Sci U S A. 2006; 103:4505–4510. [PubMed: 16537363]
25. Madhala-Levy D, Williams VC, Hughes SM, Reshef R, Halevy O. Cooperation between Shh and IGF-I in promoting myogenic proliferation and differentiation via the MAPK/ERK and PI3K/Akt pathways requires Smo activity. J Cell Physiol. 2012; 227:1455–1464. [PubMed: 21618536]
26. Yoo YA, Kang MH, Lee HJ, et al. Sonic hedgehog pathway promotes metastasis and lymphangiogenesis via activation of Akt, EMT, and MMP-9 pathway in gastric cancer. Cancer Res. 2011; 71:7061–7070. [PubMed: 21975935]
27. Ogino S, Stampfer M. Lifestyle factors and microsatellite instability in colorectal cancer: the evolving field of molecular pathological epidemiology. J Natl Cancer Inst. 2010; 102:365–367. [PubMed: 20208016]
28. Ogino S, Chan AT, Fuchs CS, Giovannucci E. Molecular pathological epidemiology of colorectal neoplasia: an emerging transdisciplinary and interdisciplinary field. Gut. 2011; 60:397–411. [PubMed: 21036793]
29. Liao X, Lochhead P, Nishihara R, et al. Aspirin use, tumor PIK3CA mutation, and colorectal-cancer survival. N Engl J Med. 2012; 367:1596–1606. [PubMed: 23094721]
30. Yamauchi M, Morikawa T, Kuchiba A, et al. Assessment of colorectal cancer molecular features along bowel subsites challenges the conception of distinct dichotomy of proximal versus distal colorectum. Gut. 2012; 61:847–854. [PubMed: 22427238]
31. Chan AT, Ogino S, Fuchs CS. Aspirin and the risk of colorectal cancer in relation to the expression of COX-2. N Engl J Med. 2007; 356:2131–2142. [PubMed: 17522398]
32. Kawasaki T, Nosho K, Ohnishi M, et al. IGFBP3 promoter methylation in colorectal cancer: relationship with microsatellite instability, CpG island methylator phenotype, and p53. Neoplasia. 2007; 9:1091–1098. [PubMed: 18084616]
33. Baba Y, Nosho K, Shima K, et al. Phosphorylated AKT expression is associated with PIK3CA mutation, low stage, and favorable outcome in 717 colorectal cancers. Cancer. 2011; 117:1399–1408. [PubMed: 21425139]
34. Morikawa T, Kuchiba A, Yamauchi M, et al. Association of CTNNB1 (beta-catenin) alterations, body mass index, and physical activity with survival in patients with colorectal cancer. JAMA. 2011; 305:1685–1694. [PubMed: 21521850]
35. Dierks C, Grbic J, Zirlik K, et al. Essential role of stromally induced hedgehog signaling in B-cell malignancies. Nature medicine. 2007; 13:944–951.

36. Riobo NA, Saucy B, Dilizio C, Manning DR. Activation of heterotrimeric G proteins by Smoothened. Proc Natl Acad Sci U S A. 2006; 103:12607–12612. [PubMed: 16885213]
37. Gerber AN, Wilson CW, Li YJ, Chuang PT. The hedgehog regulated oncogenes Gli1 and Gli2 block myoblast differentiation by inhibiting MyoD-mediated transcriptional activation. Oncogene. 2007; 26:1122–1136. [PubMed: 16964293]
38. Liao X, Siu MK, Au CW, et al. Aberrant activation of hedgehog signaling pathway in ovarian cancers: effect on prognosis, cell invasion and differentiation. Carcinogenesis. 2009; 30:131–140. [PubMed: 19028702]
39. Imamura Y, Morikawa T, Liao X, et al. Specific mutations in KRAS codons 12 and 13, and patient prognosis in 1075 BRAF wild-type colorectal cancers. Clin Cancer Res. 2012; 18:4753–4763. [PubMed: 22753589]
40. Ogino S, Kawasaki T, Kirkner GJ, Loda M, Fuchs CS. CpG island methylator phenotype-low (CIMP-low) in colorectal cancer: possible associations with male sex and KRAS mutations. J Mol Diagn. 2006; 8:582–588. [PubMed: 17065427]
41. Ogino S, Nosho K, Kirkner GJ, et al. CpG island methylator phenotype, microsatellite instability, BRAF mutation and clinical outcome in colon cancer. Gut. 2009; 58:90–96. [PubMed: 18832519]
42. Liao X, Morikawa T, Lochhead P, et al. Prognostic role of PIK3CA mutation in colorectal cancer: cohort study and literature review. Clin Cancer Res. 2012; 18:2257–2268. [PubMed: 22357840]
43. Hinoue T, Weisenberger DJ, Lange CP, et al. Genome-scale analysis of aberrant DNA methylation in colorectal cancer. Genome Res. 2012; 22:271–282. [PubMed: 21659424]
44. Ogino S, Kawasaki T, Brahmandam M, et al. Precision and performance characteristics of bisulfite conversion and real-time PCR (MethyLight) for quantitative DNA methylation analysis. J Mol Diagn. 2006; 8:209–217. [PubMed: 16645207]
45. Ogino S, Kawasaki T, Nosho K, et al. LINE-1 hypomethylation is inversely associated with microsatellite instability and CpG island methylator phenotype in colorectal cancer. Int J Cancer. 2008; 122:2767–2773. [PubMed: 18366060]
46. Ogino S, Nosho K, Kirkner GJ, et al. A cohort study of tumoral LINE-1 hypomethylation and prognosis in colon cancer. J Natl Cancer Inst. 2008; 100:1734–1738. [PubMed: 19033568]
47. Ogino S, Goel A. Molecular classification and correlates in colorectal cancer. J Mol Diagn. 2008; 10:13–27. [PubMed: 18165277]
48. Cancer Genome Atlas N. Comprehensive molecular characterization of human colon and rectal cancer. Nature. 2012; 487:330–337. [PubMed: 22810696]
49. Wang D, Xia D, Dubois RN. The Crosstalk of PTGS2 and EGF Signaling Pathways in Colorectal Cancer. Cancers. 2011; 3:3894–3908. [PubMed: 24213116]
50. Bertrand FE, Angus CW, Partis WJ, Sigounas G. Developmental pathways in colon cancer: Crosstalk between WNT, BMP, Hedgehog and Notch. Cell Cycle. 2012; 11:4344–4351. [PubMed: 23032367]
51. Leggett B, Whitehall V. Role of the serrated pathway in colorectal cancer pathogenesis. Gastroenterology. 2010; 138:2088–2100. [PubMed: 20420948]
52. Rosty C, Hewett DG, Brown IS, Leggett BA, Whitehall VL. Serrated polyps of the large intestine: current understanding of diagnosis, pathogenesis, and clinical management. Journal of gastroenterology. 2013; 48:287–302. [PubMed: 23208018]
53. Domingo E, Ramamoorthy R, Oukrif D, et al. Use of multivariate analysis to suggest a new molecular classification of colorectal cancer. J Pathol. 2013; 229:441–448. [PubMed: 23165447]
54. Hayashi Y, Molina JR, Hamilton SR, Georgescu MM. NHERF1/EBP50 is a new marker in colorectal cancer. Neoplasia. 2010; 12:1013–1022. [PubMed: 21170265]
55. Soerjomataram I, Thong MS, Korfage IJ, et al. Excess weight among colorectal cancer survivors: target for intervention. Journal of gastroenterology. 2012; 47:999–1005. [PubMed: 22426635]
56. Wu KL, Huang EY, Jhu EW, et al. Overexpression of galectin-3 enhances migration of colon cancer cells related to activation of the K-Ras-Raf-Erk1/2 pathway. Journal of gastroenterology. 2013; 48:350–359. [PubMed: 23015305]
57. Engelman JA. Targeting PI3K signalling in cancer: opportunities, challenges and limitations. Nat Rev Cancer. 2009; 9:550–562. [PubMed: 19629070]

58. van den Brink GR, Bleuming SA, Hardwick JC, et al. Indian Hedgehog is an antagonist of Wnt signaling in colonic epithelial cell differentiation. Nat Genet. 2004; 36:277–282. [PubMed: 14770182]
59. Toyota M, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB, Issa JP. CpG island methylator phenotype in colorectal cancer. Proc Natl Acad Sci U S A. 1999; 96:8681–8686. [PubMed: 10411935]
60. Beggs AD, Jones A, El-Bahrawy M, Abulafi M, Hodgson SV, Tomlinson IP. Whole-genome methylation analysis of benign and malignant colorectal tumours. J Pathol. 2013; 229:697–704. [PubMed: 23096130]
61. Yang Q, Dong Y, Wu W, et al. Detection and differential diagnosis of colon cancer by a cumulative analysis of promoter methylation. Nature communications. 2012; 3:1206.
62. Dahlin AM, Palmqvist R, Henriksson ML, et al. The Role of the CpG Island Methylator Phenotype in Colorectal Cancer Prognosis Depends on Microsatellite Instability Screening Status. Clin Cancer Res. 2010; 16:1845–1855. [PubMed: 20197478]
63. Zlobec I, Bihl M, Foerster A, Rufle A, Lugli A. Comprehensive analysis of CpG Island Methylator Phenotype (CIMP)-high, -low, and -negative colorectal cancers based on protein marker expression and molecular features. J Pathol. 2011; 225:336–343. [PubMed: 21660972]
64. Lao VV, Grady WM. Epigenetics and colorectal cancer. Nat Rev Gastroenterol Hepatol. 2011; 8:686–700. [PubMed: 22009203]
65. Hughes LA, Khalid-de Bakker CA, Smits KM, et al. The CpG island methylator phenotype in colorectal cancer: Progress and problems. Biochim Biophys Acta. 2012; 1825:77–85. [PubMed: 22056543]
66. Price TJ, Hardingham JE, Lee CK, et al. Impact of KRAS and BRAF Gene Mutation Status on Outcomes From the Phase III AGITG MAX Trial of Capecitabine Alone or in Combination With Bevacizumab and Mitomycin in Advanced Colorectal Cancer. J Clin Oncol. 2011; 29:2675–2682. [PubMed: 21646616]
67. Ogino S, Shima K, Meyerhardt JA, et al. Predictive and prognostic roles of BRAF mutation in stage III colon cancer: results from intergroup trial CALGB 89803. Clin Cancer Res. 2012; 18:890–900. [PubMed: 22147942]
68. Samowitz WS, Sweeney C, Herrick J, et al. Poor survival associated with the BRAF V600E mutation in microsatellite-stable colon cancers. Cancer Res. 2005; 65:6063–6069. [PubMed: 16024606]
69. Lochhead P, Kuchiba A, Imamura Y, et al. Microsatellite instability and BRAF mutation testing in colorectal cancer prognostication. J Natl Cancer Inst. 2013; 105:1151–1156. [PubMed: 23878352]

Synopsis

Our data reveal novel potential associations between the hedgehog, the WNT/CTNNB1, and the PI3K/AKT pathways, supporting pivotal roles of SMO and hedgehog signaling in pathway networking. SMO expression in colorectal cancer may interact with tumor CIMP status to affect patient prognosis, although confirmation by future studies is needed.

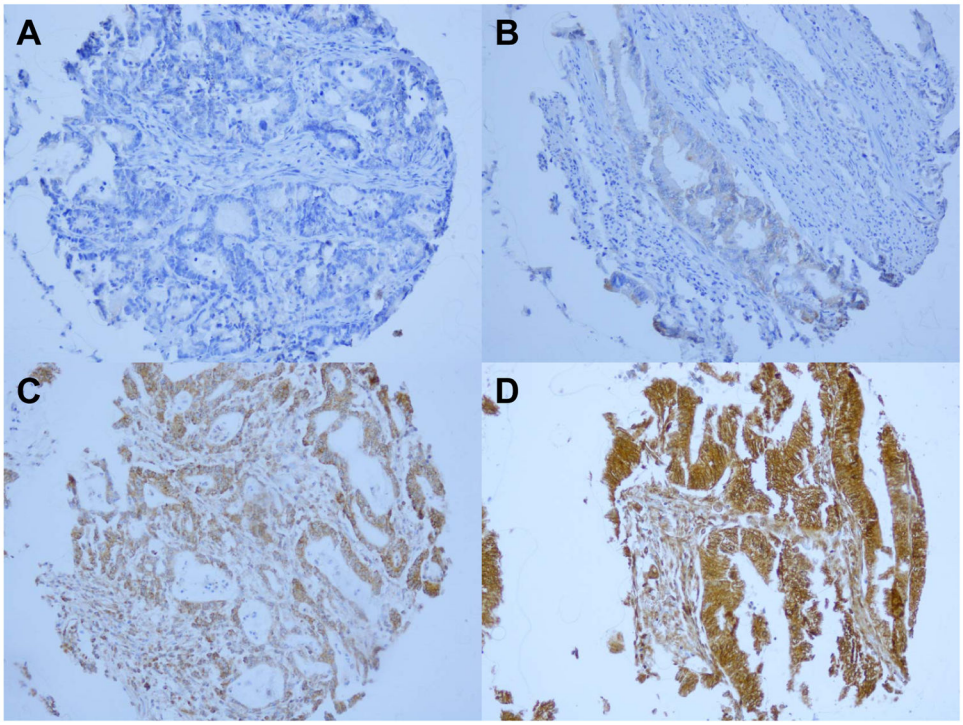

Figure 1.

SMO expression in colorectal cancer. No expression (A), weak expression (B), moderate expression (C), and intense expression (D) in colorectal cancer cells.

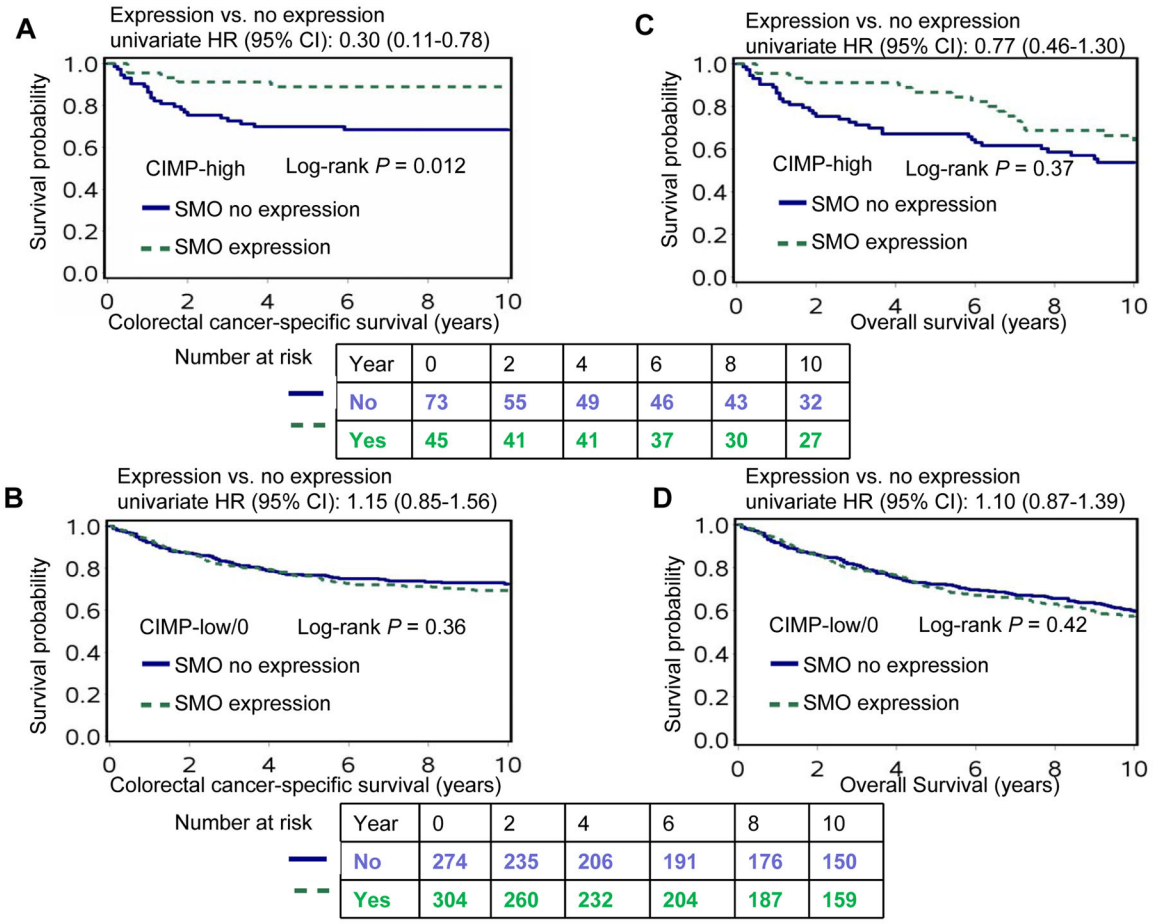

Figure 2.

Colorectal cancer-specific and overall survival in patients with colorectal cancer according to SMO expression status in strata of CIMP status. CI, confidence interval; HR, hazard ratio.

Table 1

Clinical, pathological and molecular features of colorectal cancer according to SMO expression status

Feature	Total		SMO non-expression		SMO expression		*P* value
Total No.	735		365		370		
Sex							0.093
Male (HPFS)	271	37%	146	40%	125	34%	
Female (NHS)	464	63%	219	60%	245	66%	
Mean age at diagnosis (years) ± SD	67.2 ± 8.4		67.4 ± 8.1		67.0 ± 8.7		0.60
Family history of colorectal cancer in first-degree relatives							0.20
Absent	587	80%	299	82%	288	78%	
Present	148	20%	66	18%	82	22%	
Body mass index (kg/m^2)							0.31
<30	591	81%	299	82%	292	79%	
≥30	142	19%	65	18%	77	21%	
Tumor location							0.60
Cecum	129	18%	64	18%	65	17%	
Ascending colon	156	21%	78	22%	78	21%	
Transverse colon	75	10%	41	11%	34	9%	
Descending colon	59	8%	34	9%	25	7%	
Sigmoid colon	165	23%	77	21%	88	24%	
Rectum	148	20%	68	19%	80	22%	
Disease stage							0.46
I	158	21%	82	22%	76	20%	
II	227	31%	108	30%	119	32%	
III	207	28%	96	26%	111	30%	
IV	102	14%	55	15%	47	13%	
Unknown	41	6%	24	7%	17	5%	
Tumor grade							0.044
Low	664	90%	321	88%	343	93%	
High	70	10%	43	12%	27	7%	
MSI status							0.087

Feature	Total		SMO non-expression		SMO expression		*P* value
MSI-low/MSS	602	84%	288	81%	314	86%	
MSI-high	118	16%	67	19%	51	14%	
CIMP status							0.0035
CIMP-low/0	604	84%	284	80%	320	88%	
CIMP-high	118	16%	73	20%	45	12%	
BRAF status							0.0026
Wild-type	616	85%	293	81%	323	89%	
Mutant	109	15%	69	19%	40	11%	
KRAS status							0.0027
Wild-type	461	63%	248	69%	213	58%	
Mutant	266	37%	112	31%	154	42%	
PIK3CA status							0.92
Wild-type	556	84%	275	84%	281	84%	
Mutant	107	16%	54	16%	53	16%	
Mean LINE-1 methylation level (%) ± SD	61.3 ± 9.4		61.7 ± 10.0		61.0 ± 8.8		0.33
CTNNB1 nuclear localization							0.0005
Negative	372	53%	159	46%	213	60%	
Positive	329	47%	184	54%	145	40%	
Phosphorylated AKT expression							< 0.0001
Negative	253	37%	99	29%	154	45%	
Positive	431	63%	240	71%	191	55%	

The % number indicates the proportion of patients with a specific clinical, pathological or molecular feature among all patients, or patients with specific tumor SMO expression status.

CIMP, CpG island methylator phenotype; HPFS, Health Professionals Follow-up Study; LINE-1, long interspersed nucleotide element-1; MSI, microsatellite instability; MSS, microsatellite stable; NHS, Nurses' Health Study; SD, standard deviation.

Table 2

Multivariate logistic regression analysis to calculate adjusted odds ratio (OR) for the association of a given variable (in the left column) with SMO expression (as an outcome variable)

Variable in the final multivariate model	Multivariate OR (95% CI)	*P* value
Phosphorylated AKT expression	0.48 (0.34–0.67)	< 0.0001
CTNNB1 nuclear localization	0.48 (0.35–0.67)	< 0.0001
BRAF/*KRAS* status		
BRAF-mutation/*KRAS*-wild-type (vs. *BRAF*-wild-type/*KRAS*-wild-type)	0.49 (0.28–0.85)	0.011
BRAF-wild-type/*KRAS*-mutation (vs. *BRAF*-wild-type/*KRAS*-wild-type)	1.35 (0.96–1.89)	0.082
CIMP- high (vs. low/0)	0.59 (0.35–0.98)	0.043

The multivariate logistic regression model initially included age, sex, year of diagnosis, body mass index, tumor location, family history, microsatellite instability, CpG island methylator phenotype, *KRAS*, *BRAF* and *PIK3CA* mutation, LINE-1 methylation, CTNNB1 nuclear localization and phosphorylated AKT expression. A backward elimination with threshold of $P = 0.05$ was used to select variables in the final model. When multiple hypothesis testing was performed, the *P* value for significance was adjusted to $P = 0.0033$ (= 0.05/15) by Bonferroni correction.

CI, confidence interval; OR, odds ratio.

Table 3

SMO expression status in colorectal cancer and patient mortality in strata of CpG island methylator phenotype status

		Colorectal cancer-specific mortality				Overall mortality			
Tumor characteristics	**Total No.**	**No. of events**	**Univariate HR (95% CI)**	**Stage- stratified HR (95% CI)**	**Multivariate stage-stratified HR (95% CI)**	**No. of events**	**Univariate HR (95% CI)**	**Stage-stratified HR (95% CI)**	**Multivariate stage-stratified HR (95% CI)**
CIMP-low/0									
No expression	274	75	1 (referent)	1 (referent)	1 (referent)	131	1 (referent)	1 (referent)	1 (referent)
Expression	304	95	1.15 (0.85–1.56)	1.11 (0.82–1.51)	1.08 (0.79–1.47)	156	1.10 (0.87–1.39)	1.09 (0.86–1.38)	1.02 (0.80–1.29)
CIMP-high									
No expression	73	23	1 (referent)	1 (referent)	1 (referent)	39	1 (referent)	1 (referent)	1 (referent)
Expression	45	5	0.30 (0.11–0.78)	0.37 (0.14–0.98)	0.36 (0.13–0.95)	23	0.77 (0.46–1.30)	0.87 (0.51–1.47)	0.73 (0.43–1.23)
P for interaction (SMO expression and CIMP status)			0.0090	0.035	0.035		0.22	0.44	0.26

The multivariate, stage-stratified Cox regression model initially included age, sex, year of diagnosis, body mass index, tumor location, tumor grade, microsatellite instability, *KRAS* mutation, *BRAF* mutation, *PIK3CA* mutation, LINE-1 methylation, CTNNB1 nuclear localization and phosphorylated AKT expression. A backward elimination with a threshold of $P = 0.05$ was used to select variables in the final models.

CI, confidence interval; HR, hazard ratio.