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ORIGINAL ARTICLE

# Promoter Hypermethylation and Underexpression of Patched Homolog 1 in Pancreatic and Colorectal Cancers: A Cross-Sectional Study

**Madiha NIYAZ,[1] Mosin Saleem KHAN,[1] Aaliya SHAH,[2] Rauf Ahmad WANI,[3] Omar Javid SHAH,[4] Syed BESINA,[5] Syed MUDASSAR[1]**

[1]Department of Clinical Biochemistry, Sher-I-Kashmir Institute of Medical Sciences, Srinagar-*India*
[2]Department of Biochemistry, Sher-I-Kashmir Institute of Medical Sciences Medical College, Srinagar-*India*
[3]Department of General and Minimal Invasive Surgery, Sher-I-Kashmir Institute of Medical Sciences, Srinagar-*India*
[4]Department of Surgical Gastroenterology, Sher-I-Kashmir Institute of Medical Sciences, Srinagar-*India*
[5]Department of Pathology, Sher-I-Kashmir Institute of Medical Sciences, Srinagar-*India*

**OBJECTIVE**

Patched homolog 1 (PTCH1) - a trans-membrane protein is a tumor suppressor which negatively regulates Hedgehog signaling pathway. The present study aimed to investigate the promoter hypermethylation and protein expression pattern of PTCH1 in Pancreatic cancer (PC) and Colorectal cancer (CRC).

**METHODS**

Promoter hypermethylation of PTCH1 gene was analyzed using Methylation-Specific Polymerase Chain Reaction and protein expression pattern was studied using western blotting.

**RESULTS**

Promoter hypermethylation of the PTCH1 gene was found in 42.42% (14/33) and 55.73% (34/61) of Pancreatic and Colorectal tumor samples, respectively. A significant correlation was found between PTCH1 hypermethylation and smoking status in PC while PTCH1 hypermethylation in CRC was significantly correlated with late-stage disease and lymph node metastasis. PTCH1 protein was under expressed in 30.3% (10/33) and 50.8 % (31/61) of Pancreatic and Colorectal tumor samples, respectively. Methylation analysis of PTCH1 in Pancreatic adenocarcinoma cell line 1 (PANC-1) and Colorectal adenocarcinoma cell line (HT-29) revealed hemi methylation in PANC-1, complete methylation in HT-29 and methylation was clearly associated with loss of expression.

**CONCLUSION**

Our results indicate that epigenetic silencing of the PTCH1 promoter and concomitant loss of PTCH1 protein expression may play an important role in the development and progression of these cancers.

**Keywords:** Colorectal cancer; pancreatic cancer; patched homolog 1; promoter methylation; western blotting.


## Introduction

The hedgehog signaling pathway is a key developmental pathway whose aberrant activation is known to cause uncontrolled proliferation and has been implicated in several cancers.[1] Originally identified in Drosophila as a pathway involved in patterning of the fly body plan, its homologues were soon discovered in mammalian

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Dr. Syed MUDASSAR
Department of Clinical Biochemistry,
Sher-I-Kashmir Institute of Medical Sciences,
Srinagar-India
E-mail: syed.mudassar@skims.ac.in

systems and were associated with the development of growing embryo and reactivation in adult organs could lead to uncontrolled growth and cancer.[2] In Hedgehog signaling pathway, Patched homolog 1 (PTCH1) functions as a receptor for ligands SHH, IHH, and DHH and also works as a negative regulator by repressing downstream signaling by another protein known as Smoothened (SMO). On activation of the pathway, the SHH binds to a PTCH1 receptor due to which it releases its repression on SMO which leads to phosphorylation of SMO and its subsequent activation, in turn, activating GLI transcriptional factors which translocate into the nucleus and bring about the transcription of genes responsible for growth, cellular proliferation, and metastasis. PTCH1 is also a transcriptional target of hedgehog signaling and provides a negative feedback loop to restrict hedgehog pathway activity. In the absence of ligand binding, PTCH1 inhibits SMO and the pathway is turned off.[3,4] Accumulating evidence points PTCH1 to be a tumor suppressor which inhibits the activation of the hedgehog signaling pathway.[5] Loss of function alterations in tumor suppressors such as germline inactivating mutations, or inactivation due to promoter hypermethylation contributes to cancer formation.[6] PTCH1 performs tumor suppressor functions by promoting apoptosis and enhancing cell death through a caspase-9-dependent pathway.[7] It also acts to inhibit cell cycle at a G2/M checkpoint by binding the phosphorylated form of cyclin B1 and preventing its translocation into the nucleus thus restricting mitosis.[7,8] Loss of PTCH1 function due to inactivating mutations is frequently reported innevoid basal cell carcinoma syndrome.[9] PTCH1 promoter hyper methylation and subsequent loss of expression have been reported in Gastric cancer.[10] Remarkably, Pancreatic cancer (PC) and Colorectal cancer (CRC) are major causes of mortality and morbidity worldwide in general and particularly in Kashmir.[11] Current therapeutic strategies available are extreme and tough to patients mainly because these cancers tend to metastasize rapidly to secondary locations such as lung, bone, and liver, posing a dire threat to patient survival. Therefore new biosignatures and molecules for targeted therapeutic intervention are required. No study till date has focused on the possible role of PTCH1 in the genesis of PC and CRCs, especially from this part of the world. Therefore, in this study, we investigated the promoter hypermethylation status of PTCH1 and its expression in these cancer tissues as well as cancer cell lines to elucidate its possible role in the genesis and prognosis of these cancers.

## Materials and Methods

### Study Design

This was a cross-sectional study conducted by the Department of Biochemistry in collaboration with the Departments of General Surgery and Surgical Gastroenterology. A written informed consent was obtained from each study subject. The study was approved by the Institutional Ethical Committee of Sher-I-Kashmir Institute of Medical Sciences, Srinagar.

### Patients and Samples

Keeping power of study as 80% the sample size was calculated using the statistical software G POWER v 20.1.1. The present study included PC (n=33) and CRC (n=61) patients who underwent surgical resection of colorectal or pancreatic tumors in the Department of General Surgery and Surgical Gastroenterology, respectively, from March 2018 to September 2020. The diagnosis of PC and CRC was based on the standard histopathological criteria. All patients had 1$^{st}$-time diagnosis and did not receive any adjuvant chemo/radiotherapy. The patients having any other type of malignancy or genetic disorder were excluded from the study. Tumor tissues along with their adjacent normal tissues (controls) were taken from every enrolled cancer patient for analysis. Margins of resection were well marked.

### Cell Culture

Colorectal adenocarcinoma cell line (HT-29) and Pancreatic adenocarcinoma cell line 1 (PANC-1) cell lines were purchased from National Centre for Cell Science (Pune, India). Both the cell lines were grown in the DMEM (Sigma-Aldrich, MA), supplemented with 10% fetal bovine serum (Sigma-Aldrich, MO), and 100 units of penicillin/ml and 100 µg of streptomycin/ml (Hyclone, South Logan, UT). Cells were incubated at 37°C in a humidified $CO_2$ incubator supplemented with 5% $CO_2$ (Eppendorf Brunswick).

### DNA Extraction

DNA was extracted from the tissues and cell lines with the help of Zymo DNA extraction kit according to manufacturer's instructions (Zymo Research Corp. Irvine, CA, USA). The quality and purity of DNA were assessed by agarose gel electrophoresis and optical density measured at A260/A280.

### Methylation Specific Polymerase Chain Reaction (MS-PCR)

The methylation status of PTCH1 promoter region was determined for each patient sample using Methylation-

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Specific Polymerase Chain Reaction (MS-PCR). To this end, 1–2 µg genomic DNA isolated from PC and CRC tissues and their adjacent normal tissues as well as cell lines were modified with sodium bisulfite using the EZ DNA Methylation Kit (Zymo Research Corp. Irvine, CA, USA). The modified DNAs were immediately used for MS-PCR analysis using primers, designed, and targeted, for the promoter region of the PTCH1 gene. Each primer pair targeted the methylated and the unmethylated alleles of the promoter region; Methylated Forward: 5' AATTAAGGAGTTGTTGCGGTC-3' Methylated Reverse: 5'GCTAAACCATTCTATCCCCGTA-3', Unmethylated Forward: 5'ATTAAGGAGTTGTTGTGGTTGT-3' and Unmethylated Reverse: 5' ACTAAACCATTCTAT CCCCATA-3' producing the 125 bp and 124 bp product, respectively. PCR cycling conditions for both unmethylated and methylated primers were 95°C for 8 min, followed by 40 cycles of 95°C for 1 min, 62°C for 1 min, and 72°C for 50 s, followed by a final elongation at 72°C for 7 min. Universal methylated DNA (Sigma Aldrich, USA) was used as positive control and water as negative control.

### Protein Extraction

For protein extraction, the tissue samples and the cells harvested from the culture were washed 2-3 times with ice cold PBS by centrifuging at 7000 rpm for 5 min. This was followed by lysis using NP-40 lysis buffer (20 mM Tris-HCl; pH 8.0, 137 mM NaCl, 1% Nonidet P-40, 1% glycerol, 2 mM EDTA, 10 mM NaF, 1 mM PMSF, and protease inhibitor cocktail 10 µl/1 ml of lysis buffer). After that samples were vortexed and incubated on ice for 1 h. This was followed by centrifuging at 10,000 rpm for 20 min after which supernatant was collected to obtain protein extract. The protein concentration was determined spectrophotometrically at 595 nm using the Bradford assay.

### Antibodies

Rabbit polyclonal antibody against PTCH1 was used at 1:1000 dilution (Santa Cruz technology; USA). Rabbit monoclonal antibody against beta-actin was used at 1:1000 dilution (Cell Signaling technology; USA) and was used as loading control. The fluorescence-tagged secondary antibody was used for the final detection of protein bands (anti-rabbit IR Dye 800; dilution of 1:10000 and anti-mouse IR dye 680; dilutions of 1:20000) from LI-COR Biosciences; the USA.

### Western Blotting

After quantitation and normalization, 40 µg of protein was resolved on 12% SDS-PAGE and transferred to PVDF membrane (Millipore, USA) using a semi-dry transfer method in accordance with manufacturer protocol (Hoefer, USA). For blocking membrane was treated with blocking buffer comprising of 3% BSA in PBS for 1 h after which membrane was probed with primary antibodies overnight, washed with PBST, and probed with secondary antibodies. Fluorescence was detected using Odyssey infrared detection system (LI-COR Biosciences, USA).

### Quantification of Western Blots

Densitometric analysis for quantification of western blots was performed by Image J software (NIH, Maryland USA) to measure the amount of protein present. Each protein band was normalized with the control protein band of beta-actin.

### Statistical Analysis

Statistical tests were performed using the software SPSS 16.0 (SPSS Inc., Chicago, Illinois). Independent t-test and paired t-test were performed for continuous variables; Pearson's $\chi^2$ test, Fisher's exact test or $\chi^2$ test (trend) for discrete variables. The odds ratios (ORs) and 95% confidence intervals (CIs) were obtained using logistic regression analysis. A two-sided $p<0.05$ was considered as significant.

## Results

### Patient Characteristics

The demographic and clinicopathological details of study subjects are summarized in Table 1. For PC (n=33), the majority were adenocarcinomas. The cases included 22 (66.66%) males and 11 (33.33%) females. 24 of 33 (72.72%) subjects were >50 years and 09 of 33 (27.27%) were ≤50 years having a mean age of 64.37±6.2 years. For CRC (n = 61), all cases were adenocarcinomas. The cases included 30 (49.18%) males and 31 (50.8%) females. 36 of 61 (59.01%) subjects were >50 years and 25 of 61 (40.98%) were ≤50 years having a mean age of 53.25±15.87 years.

### Promoter Hyper Methylation of PTCH1 in CRC and PC; Correlation with Clinicopathological Characteristics

Representative pictures for MS-PCR analysis observed in PC and CRC are demonstrated in Figure 1a and b, respectively.

In PC, the PTCH1 promoter region was found to be hypermethylated in 14 out of 33 cases (42.42%). Out of 14 PC cases with hypermethylation, only 2

**Table 1** Clinico-epidemiological and clinicopathologic variables of Colorectal and Pancreatic cancer patients undertaken in this study

| Characteristics | PC n=33 (%) | CRC n=61 (%) |
|---|---|---|
| Age | | |
| >50 | 24 (72.72) | 36 (59.01) |
| ≤50 | 09 (27.27) | 25 (40.98) |
| Gender | | |
| Male | 22 (66.66) | 30 (49.18) |
| Female | 11 (33.33) | 31 (50.8) |
| Smoking | | |
| Smoker | 24 (72.72) | 23 (37.70) |
| Non smoker | 09 (27.27) | 38 (62.29) |
| Tumor location | | |
| Colon | - | 35 (57.37) |
| Rectum | - | 26 (42.62) |
| Histological types | | |
| PDAC | 28 (84.84) | - |
| MUC N | 02 (6.06) | - |
| NEU T | 03 (9.09) | - |
| Grading | | |
| WD | 18 (54.54) | 33 (54.09) |
| MD/PD | 15 (45.45) | 28 (45.90) |
| Staging | | |
| I and II | 24 (72.72) | 34 (57.37) |
| III and IV | 09 (27.27) | 27 (42.62) |
| LN metastasis | | |
| Yes | 27 (81.81) | 34 (55.73) |
| No | 06 (18.18) | 27 (44.26) |
| Dwelling | | |
| Rural | 11 (33.33) | 43 (70.49) |
| Urban | 17 (51.51) | 18 (29.5) |

PC: Pancreatic cancer; CRC: Colorectal cancer; PDAC: Pancreatic ductal adenocarcinoma; MUC N: Mucinous neoplasms; NEU T: Neuroendocrine tumours; WD: Well differentiated; MD: Moderately differentiated; PD: Poorly differentiated; LN: Lymph node

(14.28%) samples showed both methylated and unmethylated bands in the tumor tissues. Table 2 describes the relationship between PTCH1 hypermethylation and clinicopathological parameters in PC. Smokers had a higher incidence of PTCH1 hypermethylation compared to non-smokers (p=0.02, OR=0.1, 95% CI=0.01-0.98). None of the other parameters of PC cases such as age, gender, histological types, grade, stage, and lymph node (LN) metastasis showed any significant association with PTCH1 promoter hyper methylation (Table 2).

In CRC, the promoter region was found to be hypermethylated in 34 out of 61 cases (55.73%). Out of 34 CRC cases with hypermethylation, 02 (5.88%) samples showed both methylated and unmethylated bands in the tumor tissues. Table 2 summarizes the correlation between promoter hypermethylation and various clinicopathological parameters of CRC patients. We found that CRC patients who were smokers were more prone to PTCH1 hypermethylation than those of non-smokers and the association was statistically significant (p=0.001, OR=7.2, 95% CI=2.0-25.6). Comparing with different stages PTCH1 hypermethylation was found more often in III and IV stages compared to I and II stages (p=0.04, OR=0.3, 95% CI=0.1-0.9). A similar significant association was also observed for LN metastasis as the majority of patients with LN metastasis had hypermethylated PTCH1 promoter (p=0.03, OR=1.2, 95% CI=0.4-3.1). None of the other studied clinicopathological parameters of CRC patients such as age, gender, tumor location, grading, and dwelling showed any significant association with PTCH1 promoter hypermethylation.

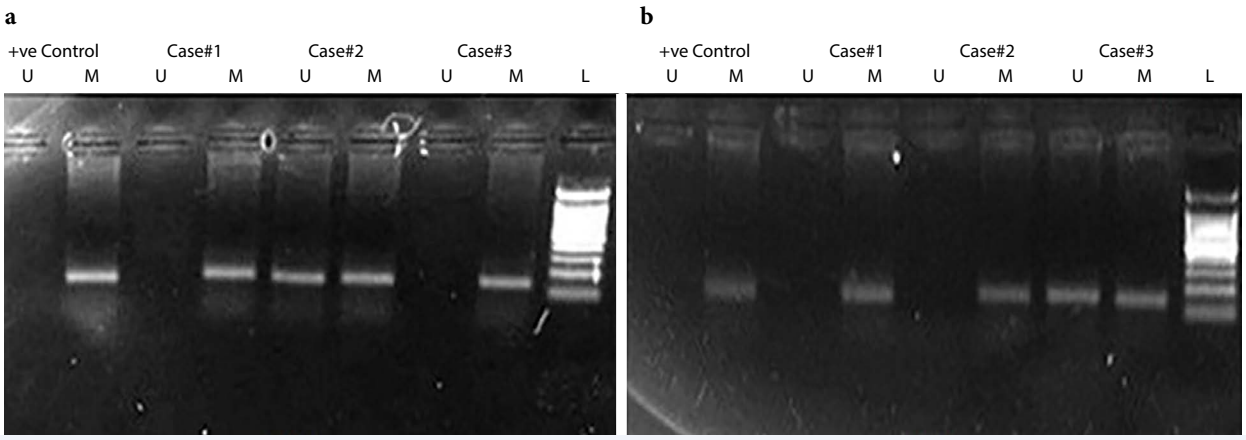


**Fig. 1.** Representative picture of (a) PTCH1 promoter hypermethylation of PC tissue samples by MSP (b) PTCH1 promoter hypermethylation of CRC tissue samples by MSP. L: 100 bp DNA marker, U: (124 bp) indicates the presence of unmethylated PTCH1, M: (125 bp) indicates the presence of methylated PTCH1.
PTCH1: Patched homolog 1; PC: Pancreatic cancer; CRC: Colorectal cancer; MSP: Methylation Specific PCR.

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**Table 2** PTCH1 promoter methylation status in PC and CRC patients and correlation with clinicopathological variables

| | PC (n=33) | | | | CRC (n=61) | | | |
|---|---|---|---|---|---|---|---|---|
| | **Methylation absent 34 (57.6%)** | **Methylation Present 14 (42.4%)** | **OR (95%CI)** | **p** | **Methylation absent 27 (44.3%)** | **Methylation present 34 (55.7%)** | **OR (95%CI)** | **p** |
| Age | | | | | | | | |
| >50 | 13 (54.16) | 11 (45.83) | 0.6 (0.1-2.9) | 0.4 | 13 (36.11) | 23 (63.88) | 2.2 (0.8-6.3) | 0.1 |
| ≤50 | 06 (66.66) | 03 (33.33) | | | 14 (56.0) | 11 (44.0) | | |
| Gender | | | | | | | | |
| Male | 10 (45.45) | 12 (54.54) | 3.7 (0.6-21.5) | 0.1 | 13(43.33) | 17 (56.66) | 1.07 (0.4-2.9) | 0.8 |
| Female | 09 (18.18) | 02 (18.18) | | | 14 (45.16) | 17 (54.83) | | |
| Smoking | | | | | | | | |
| Smoker | 11 (45.83) | 13 (54.16) | 0.1 (0.01-0.98) | **0.02** | 04 (17.39) | 19 (82.60) | 7.2 (2.0-25.6) | **0.001** |
| Non smoker | 08 (88.88) | 01 (11.11) | | | 23 (60.52) | 15 (39.47) | | |
| Histological types | | | | | | | | |
| PDAC | 15 (53.57) | 13 (46.42) | 0.4 (0.03-4.0) | 0.4 | - | - | - | - |
| MUC N | 02 (100) | 00 (00) | 0.5 (0.04-7.1) | 0.5 | - | - | | |
| NEU T | 02 (66.66) | 01 (33.33) | | | - | - | | |
| Tumor location | | | | | | | | |
| Colon | - | - | - | - | 17 (48.57) | 18 (51.42) | 0.6 (0.2-1.8) | 0.43 |
| Rectum | - | - | | | 10 (38.46) | 16 (61.53) | | |
| Grading | | | | | | | | |
| WD | 10 (55.55) | 08 (44.44) | 0.8 (0.2-3.3) | 0.6 | 16 (48.48) | 17 (51.51) | 0.6 (0.2-1.9) | 0.4 |
| MD/PD | 09 (60.00) | 06 (40.00) | | | 11 (39.28) | 17 (60.71) | | |
| Staging | | | | | | | | |
| I and II | 16 (66.66) | 08 (33.33) | 4.0 (0.7-20.3) | 0.09 | 19 (55.88) | 15 (44.11) | 0.3 (0.1-0.9) | **0.04** |
| III and IV | 03 (33.33) | 06 (66.66) | | | 08 (29.62) | 19 (70.37) | | |
| LN metastasis | | | | | | | | |
| Yes | 16 (59.25) | 11 (40.74) | 1.4 (0.2-8.5) | 0.5 | 11 (32.35) | 23 (67.64) | 1.2 (0.4-3.1) | **0.03** |
| No | 03 (50.00) | 03 (50.00) | | | 16 (37.20) | 11 (40.74) | | |
| Dwelling | | | | | | | | |
| Rural | 09 (69.23) | 04 (30.76) | 2.2 (0.5-9.7) | 0.2 | 17 (39.53) | 26 (60.46) | 1.9 (0.6-5.8) | 0.2 |
| Urban | 10 (50.00) | 10 (50.00) | | | 10 (55.55) | 08 (44.44) | | |

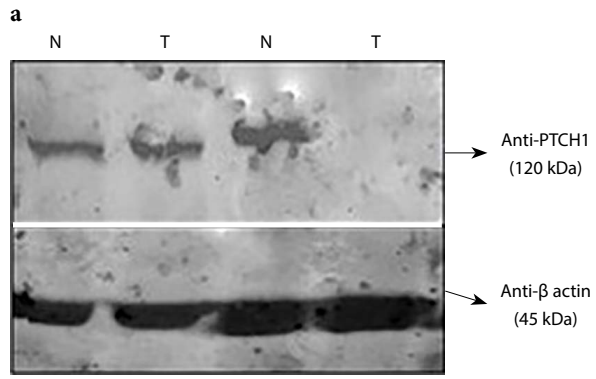
The p values <0.05 are written in bold which indicates statistical significance. PTCH1: Patched homolog 1; PC: Pancreatic cancer; CRC: Colorectal cancer; CI: Confidence interval; PDAC: Pancreatic ductal adenocarcinoma; MUC N: Mucinous neoplasms; NEU T: Neuroendocrine tumors; WD: Well differentiated; MD: Moderately differentiated; PD: Poorly differentiated; LN: Lymph node

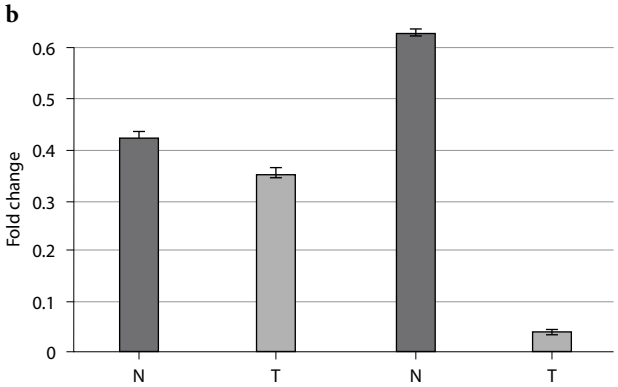
**Expression of PTCH1 Protein in CRC and PC; Correlation with Clinicopathological Characteristics**

Since promoter hypermethylation is known to influence gene expression, we examined the expression of the PTCH1 protein in all PC (n=33) and CRC (n=61) cases and their corresponding normal tissues by western blotting.

In PC, we observed that 10 (30.30%) cases showed reduced PTCH1 protein expression compared to the adjacent normal tissues. The representative pictures are shown in Figure 2a and b. The average fold change of PTCH1 down regulation in PC tumors as compared to their adjacent normal is shown in Figure 2c. We also correlated loss of PTCH1 expression with various clinicopathological parameters. No significant association was observed with any of the clinicopathological parameters (Table 3).

In CRC, out of 61 cases, 31 (50.81%) showed reduced PTCH1 protein expression compared to the adjacent normal tissues. The representative results are shown in Figure 3a and 3b. The average fold change of PTCH1 downregulation in CRC tumors as compared to their adjacent normal is shown in Figure 3c. When PTCH1 loss of expression was correlated with clinicopathological parameters, a significant association was observed for smoking. CRC patients who were smokers had significant loss of PTCH1 expression compared to non-smokers (p=0.001: OR=7.2, 95% CI=2.0-25.6). None of the other clinicopathological parameters showed any significant association with PTCH1 loss of expression (Table 3).



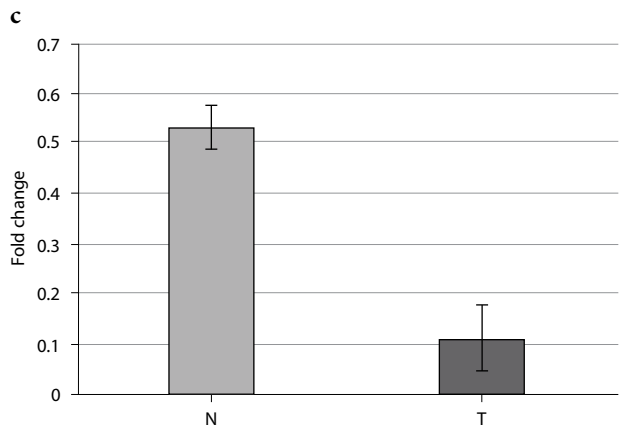




**Fig. 2.** (a) Representative Immunoblot showing PTCH1 protein (120 kDa) expression in PC tumor tissues as compared to their adjacent normal tissues, (b) normalized densitometric values of representative immunoblot, (c) the bar graph representing the average fold change of PTCH1 downregulation in PC tumors and their adjacent normal tissues at the protein level. Lane N: Normal; Lane T: Tumor. Beta actin is used as a loading control. About 60 µg protein loaded on 12% SDS-Gel transferred to PVDF membrane, anti-β actin primary Ab (1:1000), anti-PTCH1 primary ab (1:1000). LI-COR secondary Ab. The experiments were performed in triplicate, mean and S.E were calculated. Fold change using densitometry was calculated for all 33 PC samples. Average and S.E were then calculated.
PTCH1: Patched homolog 1; PC: Pancreatic cancer; PVDF: Polyvinylidene difluoride.

**Correlation of PTCH1 Hypermethylation and Expression**

Correlation of PTCH1 gene methylation and expression is shown in Table 4. Out of 14 PC cases with positive promoter hypermethylation of PTCH1, 08 (57.14%) showed reduced expression whereas in 06 (42.85%) cases protein expression was comparable to normal tissues. Of the remaining 19 PC cases without the evidence of promoter hypermethylation reduced expression was observed in only 02 (10.52%) cases and expression was normal in 17 (89.47%) cases. Overall PTCH1 promoter, hypermethylation was found to be significantly correlated with loss of protein expression (OR=0.08, 95% CI=0.01-0.5, p=0.005). Graphical representation of PTCH1 methylation and its effect on expression in PC is shown in Figure 4a.

In CRC, out of 34 cases harboring promoter hypermethylation of PTCH1, 23 (67.64%) showed reduced expression whereas in 11 (32.35%) cases protein expression comparable to normal tissues was observed. Of the remaining 27 CRC cases without the evidence of promoter hypermethylation reduced expression was observed in 08 cases and expression was normal in 19 cases. Overall PTCH1 promoter, hypermethylation was found to be significantly correlated with loss of protein expression (OR=0.2, 95% CI=0.06-0.6, p=0.003). Graphical representation of PTCH1 gene methylation and its effect on expression in CRC is shown in Figure 4b.

**Promoter Hypermethylation and Protein Expression of PTCH1 in PC and CRC Cell Lines**

PANC-1and HT-29 were evaluated for methylation and expression pattern of PTCH1. In PANC-1 cells, methylation analysis by MS-PCR revealed partial methylation. Both methylated and unmethylated bands were

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**Table 3** PTCH1 protein expression status in PC and CRC patients and correlation with clinicopathological variables

| | PC (n=33) | | | | CRC (n=61) | | | |
|---|---|---|---|---|---|---|---|---|
| | **Normal expression 23 (69.6%)** | **Loss of expression 10 (30.4%)** | **OR (95%CI)** | **p** | **Normal expression 30 (49.1%)** | **Loss of expression 31 (50.9%)** | **OR (95%CI)** | **p** |
| Age | | | | | | | | |
| >50 | 19 (79.16) | 05 (20.83) | 0.2 (0.04-1.08) | 0.06 | 15 (41.66) | 21 (58.33) | 1.5 (0.9-2.7) | 0.1 |
| ≤50 | 04 (44.44) | 05 (55.55) | | | 15(60.00) | 10 (40.00) | | |
| Gender | | | | | | | | |
| Male | 17 (73.91) | 06 (26.08) | 5.7 (1.1-28.3) | 0.42 | 15 (50.00) | 15 (50.00) | 1.07 (0.4-2.9) | 0.8 |
| Female | 06 (60.00) | 04 (40.00) | | | 15(48.38) | 16 (51.61) | | |
| Smoking | | | | | | | | |
| Smoker | 16 (66.66) | 08 (33.33) | 1.7 (0.3-10.4) | 0.4 | 09 (39.13) | 14 (60.86) | 7.2 (2.0-25.6) | **0.001** |
| Non-smoker | 07 (77.77) | 02 (22.22) | | | 21(55.26) | 17 (44.73) | | |
| Histological types | | | | | | | | |
| PDAC | 18 (64.28) | 10 (35.71) | 1.7 (0.2-18.8) | 0.4 | - | - | - | - |
| MUC N | 02 (100) | 00 (00.00) | 2.3 (0.2-23.4) | 0.5 | - | - | | |
| NEU T | 03 (100) | 00 (00.00) | | | - | - | | |
| Tumor location | | | | | | | | |
| Colon | - | - | - | - | 19 (54.28) | 16 (45.71) | 0.6 (0.2-1.8) | 0.6 |
| Rectum | - | - | | | 11 (42.30) | 15(57.69) | | |
| Grading | | | | | | | | |
| WD | 14 (77.77) | 04 (22.22) | 0.4 (0.09-1.9) | 0.2 | 15 (45.45) | 18 (54.54) | 0.6 (0.2-1.9) | 0.5 |
| MD/PD | 09 (60.00) | 06 (40.00) | | | 15 (53.57) | 13 (46.42) | | |
| Staging | | | | | | | | |
| I and II | 19 (79.16) | 05 (20.83) | 0.02 (0.04-1.0) | 0.06 | 19 (55.88) | 15 (44.11) | 0.3 (0.1-0.9) | 0.4 |
| III and IV | 04 (44.44) | 05 (55.55) | | | 11 (40.74) | 16 (59.25) | | |
| LN metastasis | | | | | | | | |
| Yes | 20 (74.07) | 07 (25.92) | 0.3 (0.05-2.1) | 0.2 | 16 (47.05) | 18 (52.94) | 1.2 (0.4-3.1) | 0.6 |
| No | 03 (50.00) | 03 (50.00) | | | 14 (51.85) | 13 (48.14) | | |
| Dwelling | | | | | | | | |
| Rural | 09 (69.23) | 04 (30.76) | 1.0 (0.2-4.7) | 0.6 | 22 (51.16) | 21 (48.83) | 1.9 (0.6-5.9) | 0.2 |
| Urban | 14 (70.00) | 06 (30.00) | | | 08 (44.44) | 10 (55.55) | | |

The p values < 0.05 are written in bold letters and indicate statistical significance. PTCH1: Patched homolog 1; PC: Pancreatic cancer; CRC: Colorectal cancer; CI: Confidence interval; PDAC: Pancreatic ductal adenocarcinoma; MUC N: Mucinous neoplasms; NEU T: Neuroendocrine tumors; WD: Well differentiated; MD: Moderately differentiated; PD: Poorly differentiated; LN: Lymph node

observed whereas, in HT-29 cells, complete methylation was observed (Fig. 5a). In PANC-1 cells, western blotting showed moderate protein expression which is in line with the observation of its partial methylation status at epigenetic level. Western blot analysis revealed negligible protein expression of PTCH1 in HT-29 cells (Fig. 5b, c).

## Discussion

Hedgehog signaling pathway is a crucial signal transduction mechanism with critical roles in embryonic development growth and maintenance of organs. [12,13] Epigenetic studies have found widespread complete or partial hypermethylation in CpG islands of tumor suppressor genes in cancer cells leading to their inactivation.[12] Therefore, in this study, we explored the methylation status of PTCH1 gene promoter and its effect at protein level, in PC and CRC tumor tissues and their adjacent normal tissues. To the best of our knowledge, this is the first study that has been undertaken to highlight epigenetic alterations of the PTCH1 promoter and its correlation with demographic and clinicopathological parameters of PC and CRC patients.

The sample size was smaller for PC typically as compared to CRC because most patients diagnosed were in

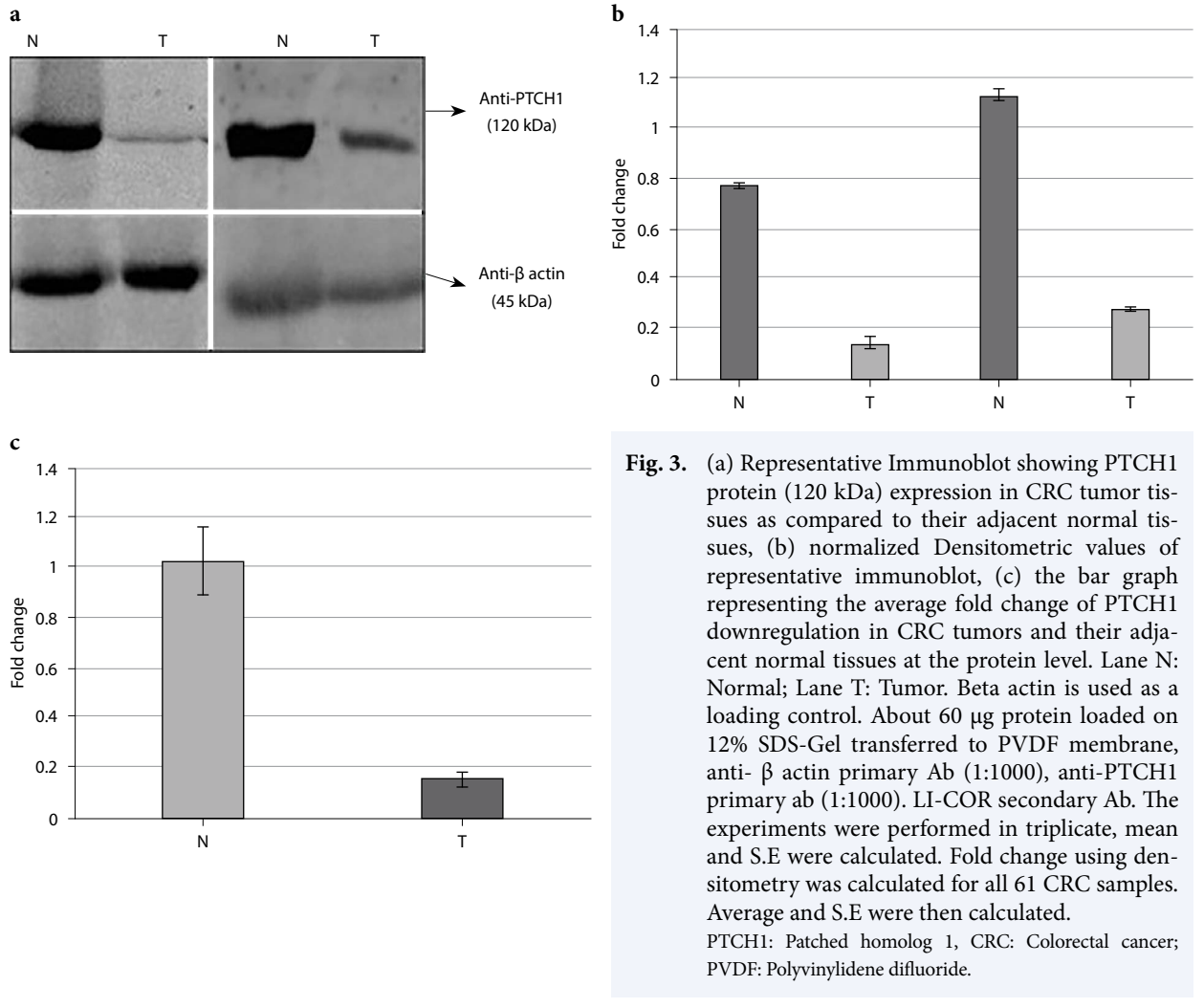


**Fig. 3.** (a) Representative Immunoblot showing PTCH1 protein (120 kDa) expression in CRC tumor tissues as compared to their adjacent normal tissues, (b) normalized Densitometric values of representative immunoblot, (c) the bar graph representing the average fold change of PTCH1 downregulation in CRC tumors and their adjacent normal tissues at the protein level. Lane N: Normal; Lane T: Tumor. Beta actin is used as a loading control. About 60 µg protein loaded on 12% SDS-Gel transferred to PVDF membrane, anti- β actin primary Ab (1:1000), anti-PTCH1 primary ab (1:1000). LI-COR secondary Ab. The experiments were performed in triplicate, mean and S.E were calculated. Fold change using densitometry was calculated for all 61 CRC samples. Average and S.E were then calculated.
PTCH1: Patched homolog 1, CRC: Colorectal cancer; PVDF: Polyvinylidene difluoride.

the terminal stage of the disease and surgically inoperable. In PC (n=33), we found that the PTCH1 gene was hypermethylated in 42.42% (14/33) of the cases. Similarly, in CRC 55.73% (34/61) had a hypermethylated PTCH1 promoter. About 14.28% and 5.88% of tumor tissues showed both methylated and unmethylated bands in PC and CRC, respectively, which could possibly be due to the admixture of normal cells, tumor heterogeneity, and/or mono-allelic methylation.

In our study, the frequency of PTCH1 promoter hypermethylation was higher in CRC (55.73%) than in PC (42.42%). The methylation status of the PTCH1 promoter in PC has never been reported so far. Only one study by Peng et al.[14] described PTCH1 methylation in aberrant crypt foci which are postulated to be the earliest known morphological precursors of CRC. Peng et al.[14] reported PTCH1 gene to be hypermethylated in 63.3% (34/54) dysplastic aberrant crypt foci which are almost comparable to our results in CRC. Epigenetic alterations of PTCH1 gene have been studied in other cancers. Cretnik et al.[15] reported that PTCH1 promoter is hypermethylated near the GLI binding site in ovarian dermoid and fibromas compared to normal ovarian tissues resulting in tumor growth. However, a study by the same authors did not observe any methylation of a PTCH1 promoter in basal cell carcinomas suggesting some other mechanism in these cancers. Moreover, PTCH1 promoter hypermethylation has been found to be a high-prevalence feature in cervical epithelial cancer.[16]

Hypermethylation of PTCH1 was found to be significantly associated with the smoking status in both PC and CRC patients. Studies in the past have reported epigenetic destruction of genome due to tobacco

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**Table 4** Correlation of PTCH1 gene methylation with PTCH1 protein expression in PC and CRC patients

| | PC | | | | CRC | | | |
|---|---|---|---|---|---|---|---|---|
| | **Loss of expression** | **Normal expression** | **OR (95%CI)** | **p** | **Loss of expression** | **Normal expression** | **OR (95%CI)** | **p** |
| PTCH1 Methylated | 08 | 06 | 0.08 (0.01-0.5) | **0.005** | 23 | 11 | 0.2 (0.06-0.6) | **0.003** |
| PTCH1 Unmethylated | 02 | 17 | | | 08 | 19 | | |

The p values < 0.05 are written in bold letters and indicate the statistical significance PTCH1: Patched homolog 1; PC: Pancreatic cancer; CRC: Colorectal cancer; CI: Confidence interval

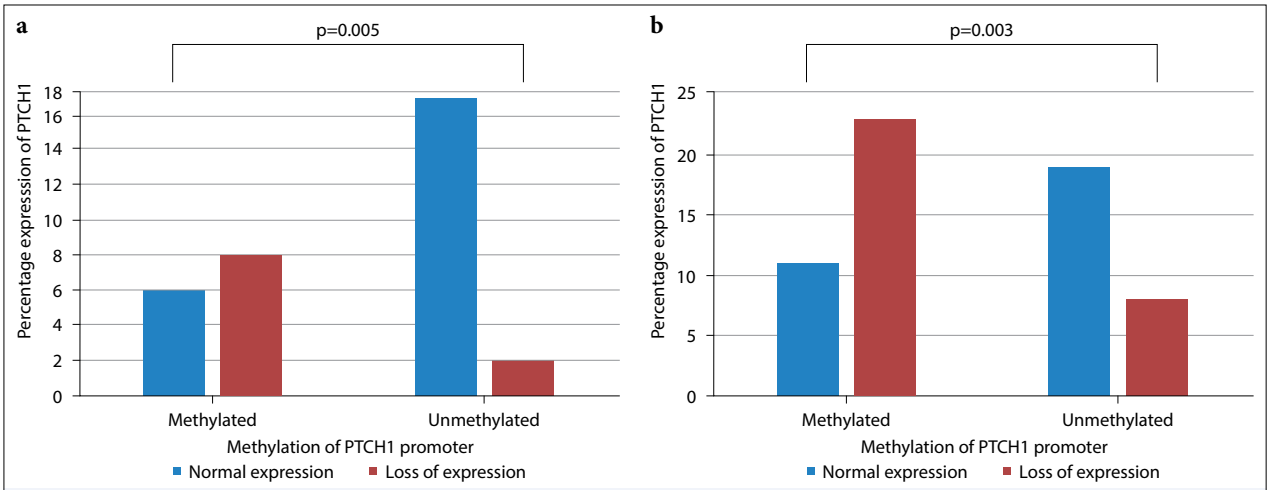


**Fig. 4.** Relationship between methylation status and expression analysis of (a) PTCH1 in PC (b) PTCH1 in CRC.
PTCH1: Patched homolog 1, PC: Pancreatic cancer, CRC: Colorectal cancer.

smoking.[17] PTCH1 promoter hypermethylation was also statistically significant with late-stage disease and LN metastasis in CRC but not in PC and these findings have been reported for the 1st time.

We observed loss of PTCH1 protein expression in 30.30% (10/33) of PC cases and 50.81% (31/61) of CRC cases. Similar pattern of low PTCH1 mRNA expression have been reported in esophageal squamous cell cancer and was related to poor prognosis.[18] Also consistent with our study, You et al.[19] reported low PTCH1 mRNA levels in metastatic CRC cells.[20] In our study, Loss of PTCH1 protein expression was associated with smoking in CRC cases therefore indicating a positive correlation between smoking and promoter hypermethylation which, in turn, leads to loss of protein expression. In cancers, promoter hypermethylation of several genes has been associated with smoking status. Cigarette smoke is considered one of the most powerful environmental modifiers of DNA methylation.[21] Cigarette smoke may modulate it through DNA damage and subsequent recruitment of DNA methyl transferases. Carcinogens in cigarette smoke, such as arsenic, chromium, formaldehyde, polycyclic aromatic hydrocarbons, and nitrosamines, can damage DNA by causing double-stranded breaks.[22,23]

In the present study, a significant correlation between PTCH1 promoter hypermethylation and loss of protein expression in both cancers was observed suggesting that PTCH1 hypermethylation has a sustainable effect on protein expression which is in line with a study in Gastric cancer wherein promoter hypermethylation of PTCH1 caused a total loss of PTCH1 protein expression.[10]

In PANC-1 cell line, hypermethylation analysis revealed a hemimethylated state with a moderate PTCH1 protein expression suggesting partial methylation does not cause total loss of expression. In PC the sample size for our study was small. Furthermore, a hemimethylated state was detected in PANC-1 cell line; therefore, further studies with larger sample size are clearly needed to strengthen our understanding of PTCH1 hypermethylation in PCs.

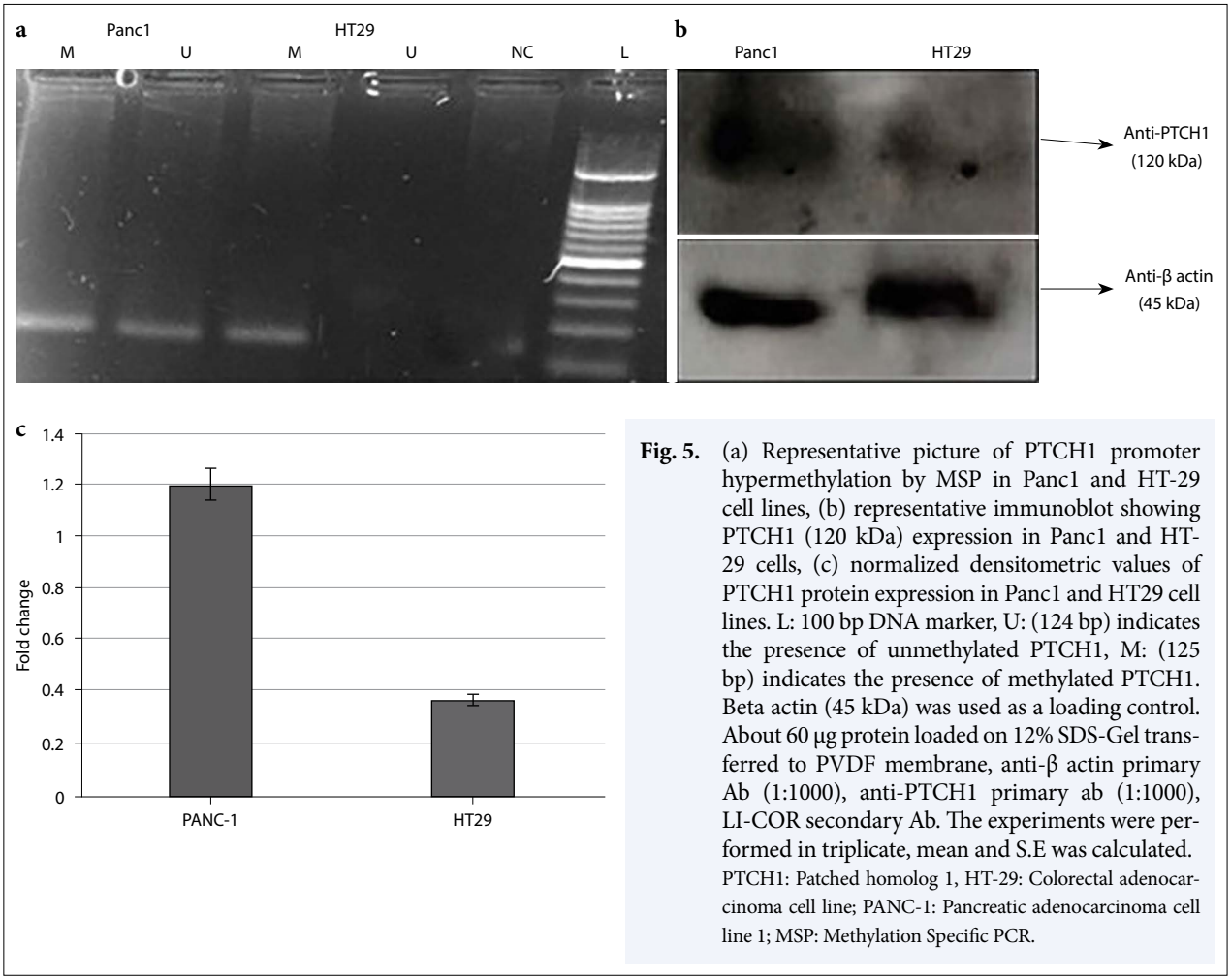


**Fig. 5.** (a) Representative picture of PTCH1 promoter hypermethylation by MSP in Panc1 and HT-29 cell lines, (b) representative immunoblot showing PTCH1 (120 kDa) expression in Panc1 and HT-29 cells, (c) normalized densitometric values of PTCH1 protein expression in Panc1 and HT29 cell lines. L: 100 bp DNA marker, U: (124 bp) indicates the presence of unmethylated PTCH1, M: (125 bp) indicates the presence of methylated PTCH1. Beta actin (45 kDa) was used as a loading control. About 60 µg protein loaded on 12% SDS-Gel transferred to PVDF membrane, anti-β actin primary Ab (1:1000), anti-PTCH1 primary ab (1:1000), LI-COR secondary Ab. The experiments were performed in triplicate, mean and S.E was calculated.
PTCH1: Patched homolog 1, HT-29: Colorectal adenocarcinoma cell line; PANC-1: Pancreatic adenocarcinoma cell line 1; MSP: Methylation Specific PCR.

In HT-29 cell line, PTCH1 promoter hypermethylation with loss of PTCH1 protein expression was observed. Similar observation was found in Breast cancer cell line (MCF-7), Breast cancer samples, Gastric cancer cell line (ASG), and Gastric cancer tissues wherein PTCH1 promoter was found to be hypermethylated with loss of PTCH1 mRNA levels. [10,24] Our observation consolidates the hypothesis that promoter hypermethylation plays a strong role in loss of protein expression as is evident from previous studies where treatment with demethylating agent (5-Aza-dC) caused significant upregulation of PTCH1 m RNA levels in breast cancer and gastric cancer cell lines.[10,24]

### Limitations of the Study

Considerable number of patients in this region was diagnosed with advanced stage PC and was in-operable. For that reason, the tumor tissue could not be collected from those very patients. Therefore, studies are warranted to validate the results, especially in case of PC, due to relatively modest sample size.

## Conclusion

Our results suggest that PTCH1 gene inactivation by promoter hypermethylation, followed by significant loss of PTCH1 at protein level, may be a frequent molecular event in PC and CRC, hence, may play a role in the progression of these cancers. This study supports the role of PTCH1 as a tumor suppressor gene in PC and CRC.

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**Conflict of Interest:** All authors declared no conflict of interest.

**Ethics Committee Approval:** The study was approved by the Institutional Ethics Committee Sher-I-Kashmir Institute of Medical Sciences Srinagar having Institutional Review Board (IRB) (No: SIMS 1 131/IEC-SKIMS/2015-183, Date: 03/09/2015).

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