Original article

Hypomethylation of Sonic hedgehog in colorectal cancer

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Abstract:

Background: Past research has demonstrated that a changed Sonic Hedgehog (SHH) pattern contributes to the development of colorectal cancer. This study's objective was to determine whether the SHH gene's promoter hypomethylation might be used to predict the likelihood of developing colorectal cancer. ***Methods:*** For the current investigation, 50 newly diagnosed and untreated colorectal cancer patients' tumor samples and surrounding non-tumor tissues were gathered. SHH methylation was determined by methylation-specific PCR (MSP) and results correlated with several studied clinicopathological parameters. ***Results:*** Our results showed that SHH methylation levels are significantly present in normal tissues as compared to tumor tissues. ***Conclusion:*** We conclude SHH promoter is hypomethylated in colorectal cancers which may have an effect driving normal colon and rectum cells to cancer.

Keyword: Hypomethylation; Sonic Hedgehog; Methylation specific PCR; Colorectal Cancer.

Bangladesh Journal of Medical Science Vol. 23 No. 01 January'24 Page : 200-206
DOI: https://doi.org/10.3329/bjms.v23i1.70719

Introduction:

Colorectal cancer is a deadly malignancy with worldwideoccurrence[1]CRC over the years has been extensively studied concerning molecular markers to develop personalized treatment options[2]. Epigenetics during the last decade has emerged as a crucial element involved in regulation mechanisms and genome-wide DNA hypo methylation is an important epigenetic alteration in several malignancies including CRC. It is considered to be one of the initiating events in carcinogenesis and contributes to changing proteomics in the cells[3,4]

The SHH signaling system is crucial for carcinogenesis, maintaining healthy adult tissues, and embryogenesis. The pancreas, intestines, and stomach are only a few of the tissues that express the SHH protein, which is the key protein in this system. Through the binding of SHH to the transmembrane receptor protein Patched, the SHH signaling cascade is known to speed up cell development and inhibit apoptosis (PTCH1). SHH's inhibition of Smoothened (another protein) is released upon binding to PTCH1, which subsequently activates the transcription factors GLI proteins. Once in the nucleus, the GLI proteins cause transcription of the genes necessary for cell growth and proliferation..[5,6]

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It is well-recognized that hedgehog signaling is crucial for the normal growth and maintenance of colon cells and its aberrant activation has been strongly linked to several malignancies. The SHH signaling system may be activated and play a significant role in the development, spread, invasion, and maintenance of malignancies, according to mounting data throughout time.[7]

Breast, stomach, and pancreatic cancers have all been linked to abnormal activation of the SHH system, which is mostly caused by overexpression of SHH proteins (Niyaz et al. 2019; Bailey et al. 2008; Noman et al. 2016) Numerous studies have demonstrated that increased expression of SHH is required to sustain tumor development, recurrence, metastasis, and survival of colorectal cancer cells as well as that it contributes to colon carcinogenesis (Wu et al. 2017a; Po et al. 2020)

Since the expression of a protein is influenced by the methylation status of its gene promotor therefore in this study we performed a comparative analysis of SHH methylation level in colorectal tumor and non-tumor tissues using methylation-specific PCR to determine epigenetic changes if any in the SHH gene promoter. We further compared the data with pertinent clinicopathological factors to assess any predictive or diagnostic value of SHH methylation.

Method and material:

Materials and Methods

Patients and Samples

The 50 CRC tissues that had undergone surgical resection at the SKIMS Department of General Surgery between March 2019 and September 2020 were included in the current study, along with the neighboring normal tissues that served as controls. All of the study participants provided their written informed permission. The SKIMS ethics committee gave the study its approval. No chemo or radiation was administered to any of the patients, who all received initial diagnoses.

DNA extraction

The Zymo DNA extraction kit was used to extract DNA from the tissues and cell lines following the manufacturer's instructions. On a 1 percent agarose gel, the DNA was measured and the integrity of the extracted DNA was examined using ethidium bromide staining.

Methylation Specific PCR (MS-PCR)

Using MS-PCR, the methylation status of the SHH promoter region was identified for each patient sample. For this, the EZ DNA Methylation Kit was used to methylate 1-2 g of genomic DNA extracted from colorectal cancer tumors and the surrounding normal tissues (Zymo Research Corp. Irvine, CA, USA). The modified DNAs were employed right away for MS-PCR analysis with primers that were specifically designed for the SHH gene promoter region. The promoter region's methylation and unmethylated alleles were targeted by each primer pair; Methylated forward:5'- AGAGTTTTTCGTAGTCGCGGC -3'

Methylated Reverse:3'ATCCCCGTACGAATCCGTA CG-5'

Unmethylated Forward:5'- GGTGGAGAGTTTTTT-GTAGTTGTGGT -3' and*Unmethylated Reverse:3'- AAACTATCCCCATACAAATCCATACA -5'* producing the 169 bp and 179bp product respectively.PCR cycling conditions for both unmethylated and methylated primers were 95ºC for 8 min, followed by 40 cycles of 95ºCfor 1 min, 64ºC for 1 min, 72ºC for 50 sec and a final elongation reaction at 72ºC for 7 min. Water served as the negative control while universal methylated DNA (Sigma Aldrich) served as the positive control.

Results:

Characteristics of Study Subjects

The present study involved a total of fifty CRC cases(n=50). All the cases were histologically confirmed that underwent surgical resection at the Sher-I-Kashmir Institute of Medical Sciences (SKIMS). Patients who had radiation or chemotherapy were not included in the research. There were no age, sex, histology, or stage limitations but colorectal cancer patients simultaneously suffering from any other malignancy were excluded from the study.

The CRC cases included 36 (72%) males and 14 (28%) females. 29 of 50 (58%) subjects were >50 years and 21of 50 (42%) were ≤50 years having a mean age of 53.5±12.87. Out of 50 cases, 22 (44%) were smokers and 28 (56%) were non-smokers. 19 (38%) had Colon cancer and 31 (62%) had Rectal cancer. 45 (90%) presented with stage I or II disease

and 5 (10%) had stage III or IV disease. Based on the grade of differentiation 27 (54%) cases were well differentiated and 21 (42%) were moderately and 2 (4%) were poorly differentiated. Table 1 provides a full description of the clinicopathological features of these individuals.

Table 1: Clinico-epidemiological and clinicopathologic variables of Colorectal Cancer patients undertaken in this study:

Characteristics	Number and Percentage (%)
Age	
>50	29 (58)
≤50	21 (42)
Gender	
Male	36 (72)
Female	14 (28)
Dwelling	
Rural	29 (58)
Urban	21 (42)
Social Class	
Low	20 (40)
Middle	30 (60)
Family History	
Yes	14 (28)
No	36 (72)
Smoking Status	
Smoker	22 (44)
Non-smoker	28 (56)
Lifestyle	
Active	29 (58)
Sedentary	21 (42)
Body Mass	
Normal	21 (42)
Obese	10 (20)
Underweight	19 (38)
Salt tea Intake	
Yes	47 (94)
No	3 (6)
Red meat consumption	
Yes	47 (94)
No	3 (6)
Sundried Vegetables	
Yes	44 (88)
No	6 (12)
Junk food consumption	
Yes	2 (4)
No	48 (96)
Pesticide Exposure	
Yes	26 (52)
No	24 (48)
Site of Tumour	
Colon	19 (38)
Rectum	31 (62)
Tumour Differentiation	
Well	27 (54)
Moderate	21 (42)
Poor	2 (4)
TNM Stage	
T1	4 (8)
T2	23 (46)
T3	19 (38)
T4	4 (8)
T1+T2	27 (54)
T3+T4	23 (46)
Stage	
I+II	45 (90)
III+IV	5 (10)
Tumour Grade	
1	27 (54)
2	21 (42)
3	2 (4)

DNA Extraction

Total cellular DNA was isolated from Colorectal tumor tissue samples (n=50) and their adjacent normals. To check the integrity of DNA, the samples were run on 1 % agarose gel electrophoresis

Clinicopathological features and the SHH gene's methylation pattern in colorectal cancer

Figure 1 illustrates representative outcomes for MSP analysis seen in colorectal cancer.

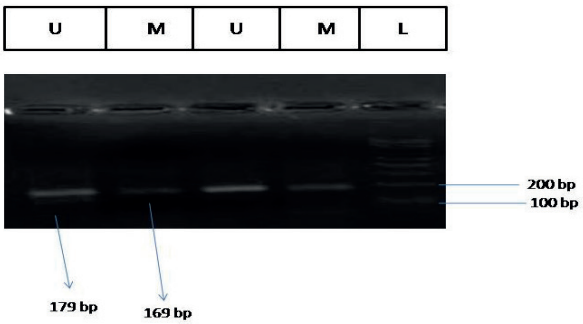

Figure 1: Representative image of *SHH* promoter Methylation pattern of CRC tissue samples by MSP.

L: 100 bp DNA marker, U: (179 bp) indicates the presence of unmethylatedSHH , M: (169bp) indicates the presence of methylated SHH

SHH promoter Methylation pattern was assessed in 50 CRC cases and their neighboring normal tissues, which histology has proven,using MSP. In tumour tissues, methylation was found to be absent in 76% (38/50) cases whereas only 24% (12/50) cases showed the presence of promoter methylation. Out of 12 CRC cases in which methylation was present 66.6% (8/12) showed both methylated and unmethylatedbands. While 33.3% (4/12) showed only methylated bands.

In the adjacent normal tissues, 80% (40/50) cases showed the presence of methylation. While in only 20% (10/50) cases no methylation was found.

Further SHH gene promoter methylation pattern was correlated with several clinicopathological characteristics such as age, gender, tumour location, tumour grading, tumour staging, smoking status, and family history. The correlation between the methylation status of the SHH promoter and numerous clinicopathological characteristics is summarized in Table 2.

We found that the methylation pattern of the SHH gene promoter did not significantly correlate with any of the investigated clinicopathological characteristics.

Discussion:

Epigenetic modification such as DNA hyper/ hypo methylation affects the expression of a wide variety of genes [8]. For tumor suppressors, these CpGhypermethylation events lead to the inactivation of critical tumor suppressor genes which then become the driving force behind cancer initiation and progression[9].CpGdemethylation or hypomethylation events are strongly associated with the initiation and progression of cancer. DNA hypomethylation is virtually always present alongside genome hypermethylation in cancer, according to high-resolution genome-wide studies. Uncontrollable growth and cancer are brought on by hypo methylation in the growth-promoting genes' promoter region (Ehrlich 2009)

Hedgehog signaling transduction has become a significant pathway that is essential for growth, tissue repair, and oncogenesis.

Alterations in hedgehog signaling genes leading to pathway malfunction have been associated with developmental defects and several cancers [10]

The SHH ligand is the pathway's essential element. It adheres to its receptor PTCH1. This binding causes the signal to be transduced to growth-promoting GLI transcription factors through a protein called SMO. Without SHH binding, PTCH1 inhibits SMO and turns off the pathway.

SHH overexpression has harmful consequences in a variety of solid malignancies, including breast, pancreatic and gastric cancers. Several studies established that aberrant hedgehog signaling due to SHH overexpression is essential for tumor growth, recurrence, metastasis, and survival of colorectal cancer cells[5]

In the current investigation, we have explored the methylation pattern of SHH promoter region in colorectal cancers by MS PCRas a possible mechanism for up-regulation of SHH in colorectal cancers and also correlated results with several clinico- pathological characteristics.

We observed that in the majority of normal colon tissues 40 out of 50 (80%) had methylated promoter regions whereas in only 10 out of 50 (20%) no methylation was present. The capacity of transcription factors to bind to the transcription start site, controlling mRNA transcription and subsequently its protein production, may be influenced by the methylation of DNA chromatin promoters in normal colon tissues.

Table 2: SHH Promoter Methylation status in patients with colorectal cancer and its relationship to clinicopathological factors.

Characteristics	Methylation Present	Methylation Absent	Odds Ratio (95%CI)	P-Value	Chi2
Age					
>50	5(17.24)	24(82.76)	0.4(0.08-1.9)	0.189	1.73
≤50	7(33.33)	14(66.67)			
Gender					
Male	10(27.78)	26(72.22)	2.3(0.4-24.5)	0.316	1.006
Female	2(14.29)	12(85.71)			
Dwelling					
Rural	7(24.14)	22(75.86)	1.01(0.2-4.8)	0.979	0.0007
Urban	5(23.81)	16(76.19)			
Social Class					
Low	4(20.00)	16(80.00)	0.7(0.1-3.2)	0.589	0.32
Middle	8(26.67)	22(73.30			
Family History					
Yes	3(21.43)	11(78.57)	0.8(0.1-4.1)	0.791	0.07
No	9(25.00)	27(75.00)			
Smoking Status					
Smoker	8(36.36)	14(63.64)	3.4(0.7-18.1)	0.070	3.32
Non-smoker	4(14.29)	24(85.71)			
Lifestyle					
Active	5(19.23)	21(80.77)	0.6(0.1-2.6)	0.411	0.67
Sedentary	7(29.17)	17(70.83)			
Body Mass					
Normal	7(33.33)	14(66.67)	2(0.3-23.8)	0.408	1.82
Obese	2(20.00)	8(80.00)	2.7(0.5-18.6)		
Underweight	3(15.79)	16(84.21)			
Salt tea Intake					
Yes	11(23.40)	36(76.60)	0.6(0.03-39.3)	0.696	0.15
No	1(33.33)	2(66.67)			
Red meat consumption					
Yes	11(23.40)	36(76.60)	0.6(0.03-39.3)	0.696	0.15
No	1(33.33)	2(66.7)			
Sundried Vegetables					
Yes	10(22.73)	34(77.27)	0.6(0.1-7.5)	0.568	0.32
No	2(33.33)	4(66.67)			
Junk food consumption					
Yes	0(0)	2(100)	0(0-6.3)	0.417	0.66
No	12(25.00)	36(75)			
Pesticide Exposure					
Yes	7(26.92)	19(73.08)	1.4(0.3-6.6)	0.614	0.25
No	5(20.83)	19(79.17)			

Characteristics	Methylation Present	Methylation Absent	Odds Ratio (95%CI)	P-Value	Chi2
Site of Tumour					
Colon	6(31.58)	13(68.42)	1.9(0.4-8.7)	0.326	0.96
Rectum	6(19.35)	25(80.65)			
Tumour Differentiation					
Well					
Moderate	5(18.52)	22(81.48)	0.6(0.1-2.7)	0.490	1.42
Poor	6(28.57)	15(71.43)	0.2(0.002-21.3)		
	1(50.00)	1(50.00)			
TNM Stage					
T1	0(0)	4(100)			
T2	6(26.09)	17(73.91)		0.711	1.38
T3	5(26.32)	14(73.68)			
T4	1(25.00)	3(75.00)			
T1+T2	6(22.22)	21(77.78)		0.750	0.10
T3+T4	6(26.09)	17(73.91)	0.8(0.2-3.7)		
Stage					
I+II	10(22.22)	35(77.78)	0.8(0.06-49.43)	0.377	0.77
III+IV	2(40.00)	3(60.00)			
Tumour Grade					
1					
2	5(17.86)	22(81.48)	0.6(0.1-2.7)		
3	6(30.00)	15(71.43		0.424	1.71
	1(50.00)	1(50.00)	0.2(0.002-21.3)		

When colorectal cancer tissues were analyzed it was observed that 38 out of 50 (76%) CRC tissues had no methylation present i.e. were hypo methylated and only 12 of 50 (24%) had methylation present. In our study majority of colorectal cancer tissues, 76% were hypo methylated. The hypomethylation in colorectal cancer tissues might be a mechanism underlying the increased expression of SHH in colorectal cancers which may be an important event in colon carcinogenesis. The same outcomes have also been seen by Fu *et al* where they found SHH promoters to be hypo methylated in colon cancers. [11]. SHH protein was identified as being overexpressed in breast cancer tissues and promoter hypo methylation was present in breast cancer tissues [12]. Methylation of the SHH promoter region has been found to be common in normal gastric pit cells but very rare in gastritis, intestinal metaplasia, dysplasia, and carcinoma [13]. Additionally, the expression of SHH protein was increased while promoter methylation was reduced in breast cell lines treated with the 5-Aza demethylating agent. All these studies provide support to our study that promoter hypo methylation of the SHH gene is an important feature in colon carcinogenesis. Several other tumor-associated genes are also reported to be hypomethylated in colorectal cancers causing their increased expression supporting the data observed with SHH [14–16]. There was no significant link between any of the variables in our investigation promoter hypo methylation and any clinicopathological parameters such as age, staging, or tumor differentiation. One of the reasons for that can be the considerably small sample size in our study. No significant study in the past has correlated hypo methylation of SHH with clinicopathological characteristics whereas Cui et al correlated the expression of SHH with several clinicopathological characteristics and found a statistically significant association with early-stage disease [12].

Conclusion:

In conclusion, we investigated the significance of SHH hypo methylation in colorectal cancer, as a possible underlying mechanism affecting the expression. Our findings suggest that SHH hypo methylation is present in colorectal cancers and may contribute to overexpression of SHH protein which in turn can activate unregulated hedgehog signaling contributing to cancer.

This study supports future explorations of SHH biology in colorectal cancer with a better

understanding of the regulation mechanism.

Conflicts of interest

The writers affirm that there is no conflict of interest.

Ethical clearance

A written consent was taken from all the patients prior to surgery, and they were also apprised of the ongoing study, which was approved by the Ethical Clearance Committee of SKIMS (SIMS 1131/IEC-SKIMS/2018-330).

Acknowledgments

The authors claim there are no conflicts of interest that may be seen as undermining the objectivity of the study presented. Following written patient permission, tissue samples were taken after informing all patients about the study. The SKIMS Ethical Clearance Committee authorized the project. The authors gratefully acknowledge the technical staff of the Department of General Surgery and Surgical Gastroenterology who helped us in procuring the tissue samples.

Funding

The study was supported by Sher-I-Kashmir Institute of Medical Sciences, Soura, Srinagar, Kashmir 190011 India

Authors Contribution

Maheera Teli, Madiha Niyaz, Gowhar Rashid and Ishrat Parveiz has done the main experimental work and played a role in designing the study as well as drafting the manuscript, Rouf A Wani provided the samples for the study. Syed Mudassar gave concept and also provided all the Laboratory support required in accomplishing the study

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