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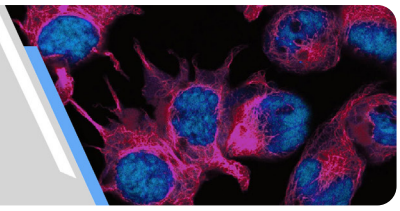

Elevated Expression of *Notch 2 & Notch 3* is associated with Disease Progression in Colorectal Cancer

Abhay Kumar Sharma[1,2], Nimisha[2], Apurva[2], Arun Kumar[2], Asgar Ali[2], Sundeep Singh Saluja^,[2,3], Birendra Prasad*,[1]

Abstract

Background: The potential involvement of Notch signaling pathway in cell fate decision, tumor heterogeneity and angiogenesis in solid tumors can be explored in colorectal cancer (CRC). This might further help to improve outcomes in CRC. Here, the promoter methylation of Notch receptor gene (*Notch2* and *Notch3*) and their co-expression with its downstream transcription factor *Hes1* has been analyzed.

Methods: Seventy-two CRC patients were enrolled to study the role of *Notch2*, *Notch3* and *Hes1* genes in colorectal cancer. Promoter methylation and mRNA expression in tumor and adjoining normal tissue were assessed by Methylation Specific PCR and quantitative Real time PCR respectively. Statistical correlation was done by using SPSS.

Results: We found that *Notch2 and Notch3* were hypomethylated in 52/72 (72.22%) and 54/72 (75%) cases respectively. Hypomethylation of *Notch 2 and Notch 3* showed significant association with advanced stage (p=0.001) and (p=0.003) and nodal metastasis (p=0.036) and (p=0.012) respectively. Both *Notch 2 and Notch 3* showed increased mRNA expression in 49 (68.05%) and 51(70.84%) patients with a fold change of 3.37 and 5.43 respectively. Positive correlation between hypomethylation and expression was observed for both genes. High expression of *Hes1* was found in 53(73.61%) of cases which was highly relatable with over expression of notch receptor genes. Upregulation of *Notch 2, Notch 3 and Hes1* showed significant association with high grade tumors, advance stage and presence of LN metastasis, additionally *Notch 3 and Hes1* showed significant association with distant metastasis.

Conclusion: Hypomethylation of *Notch 2 and 3* receptors is playing crucial role in regulating the expression of these genes in CRC. Overexpression of *Notch 2, Notch 3* and Hes1 are associated with disease progression in CRC.

Keywords: Colorectal Cancer; Hypomethylation; mRNA Expression; Notch 2; Notch 3

Introduction

Colorectal cancer (CRC) is the third most common cancer comprising 10 % of the newly diagnosed cancer cases and second leading cause of cancer related death (9.4%) worldwide (GLOBOCAN 2020). It is the second most common cancer in women and third among men worldwide [1,2]. Basic tenets of colorectal tumorigenesis are firstly the tumor develop from activation of oncogenes and deactivation of tumor suppressor genes. The genetic and/or epigenetic changes occur at multiple sites and summation of these changes

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lead to malignant transformation. Understanding of these changes is imperative to find new solutions. Hence, it is important to identify molecular markers and potential drug targets to enhance the clinical decision making for treatment. There are several molecular pathways known to be involved in the development of CRC, which includes chromosomal instability (CIN), Wnt, Hedgehog and Notch signaling pathways [3,4]. Wnt and Notch signaling occurs maximum at the base of crypt. Emerging concept that cancer stem-like cells (CSCs) play a role in both chemoresistance and tumorigenesis in various cancers including solid tumours. Its role in CRC might help in improving outcomes in colorectal cancer. The Notch signaling pathway is a highly conserved signaling system involved in various biological processes, including cell differentiation, proliferation, apoptosis and stem cell maintenance [4,5]. Moreover, Notch can function as both oncogene and tumor suppressor gene. Human Notch consists of four transmembrane Notch receptors (Notch 1-4) and five Notch ligands (Delta-like-1, Delta-like-3, Delta-like-4, Jagged-1 and Jagged-2) [6]. Notch pathway gets activated by the interaction of Notch receptors with Notch ligands leading to secretion of γ-secretase protein complex. As a result, Notch receptors undergo proteolytic cleavage to release the Notch intracellular domain (NICD) which subsequently enters into the nucleus and associates with DNA binding proteins and acts as a transcription-activating factor for the regulation of Notch target genes such as Hairy/enhancer of Split 1 (*Hes1*) [7]. The Notch signaling pathway controls the transcriptional repressor of *Hes1*, a basic helix-loop-helix (bHLH) molecule that is increased upon activation of Notch [8]. *Hes1* plays a crucial role in cellular development, sustaining intestinal progenitor cells and neural stem cells, and controlling apoptosis [9]. Abnormal activation of the Notch pathway plays an important role in tumor development and progression [10]. The upregulation of the Notch pathway results in cell proliferation, Epithelial-mesenchymal transition (EMT) and angiogenesis. The aggressiveness of CRC could be due to the active involvement of the oncogenic Notch pathway. Therefore, the possible approaches should be targeted to explore the alteration in various Notch receptors, ligands and Notch targets genes that cause activation of Notch pathway in colorectal cancer. Notch 2 is reported to be up-regulated in gastric cancer and brain tumors while its down-regulation was reported in renal cell carcinoma and non-small cell lung cancer [11–13].The significance of *Notch 2 gene* in colorectal cancer is disputed. In fact, it may play role in tumor inhibition in colon carcinogenesis as per study by Chu et al [14]. On a contrary, it could also behave as a key regulator of carcinogenesis in the Notch pathway. Moreover, the mechanism of its dysregulation is less known especially in colorectal cancer and needs more attention. *Notch 3* is found to be over-expressed in cervical cancer tissues [15], lung cancer cell line [16] and Gastric cancer [17]. No clear molecular mechanism for the activation of Notch 3 overexpression has been reported. However, increased tumorigenesis was found to be associated with abnormal methylation pattern during the multistage carcinogenesis of cervical cancer [18]. Moreover, *Notch 2 and 3* inhibitors Tarxetumab could have antitumor effects by blocking receptor activation thereby inhibiting tumor growth. Gliotoxin is a myotoxin, which acts by inhibiting the formation of DNA bound *Notch 2* complexes, and induces apoptosis in various cancers such as melanoma, hepatocellular carcinoma and pancreatic cancer [12,19,20]. These inhibitors may help in tumor reduction in cases where Notch shows overexpression. *Notch 2 and Notch 3* activation could be responsible for the elevated expression of the downstream target gene *Hes1*. Herein, we have investigated the methylation status of Notch 2 and 3 and the mRNA expression of Notch 2, 3 along with its downstream target gene *Hes1* in CRC patients. Statistical correlation between molecular evaluation and clinical-pathological parameters was also performed.

Materials and Methods

Ethical Clearance

Ethical approval for the study was obtained from Institutional Ethical Committee Maulana Azad Medical College and Associated Hospital, New Delhi with file number F.1/IEC/MAMC/85/03/2021/No.430.

Patients and Sample Collection

Tumor and adjacent normal (at least 5cm away from the tumor) tissue specimen from 72 CRC patient were collected in appropriate buffer (PBS for DNA and RNA later for RNA) who underwent treatment for CRC in the Department of Gastrointestinal Surgery, GIPMER, New Delhi. The specimen was collected from patients before obtaining any neoadjuvant treatment. Among 72 patients, 46 were male and 26 were female, with mean age of 49.03 ± 14.2 years. Informed consent was taken from all participants. Detailed clinical and demographic parameters along with their management plan were recorded for all the patients enrolled in the study.

DNA Extraction and Bisulfite Modification

Genomic DNA was isolated from the tissue specimens (tumor and adjacent normal tissues) using Wizard Genomic DNA Purification Kit (Promega USA) following the manufacturer's instructions. The purity of DNA was checked using the NanoDrop spectrophotometer (Thermo Fisher, New York) and quantified by Qubit 4 fluorometer (Invitrogen, USA). The quality of DNA was checked on 0.8% agarose gel. Bisulfite treatment of purified DNA was performed using the EZ DNA Methylation-Gold kit (Zymo Research-D5006, USA). One microgram of genomic DNA was treated with bisulfite and purified according to the manufacturer's instructions. Bisulfite treated DNA was stored at -20˚C for further analysis.

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Methylation Specific Polymerase Chain Reaction (MS-PCR)

Promoter methylation status of the *Notch 2 and 3* genes was determined using Methylation Specific PCR. Notch promoter regions were selected using the Eukaryotic promoter database (EPD) and their respective CpG islands were predicted using Meth Primer tools (Figure 1). The primer was synthesized commercially (Integrated DNA Technologies) according to the specific sequence of CpG island. The primer used in MS-PCR is listed in table 1. PCR was performed as the previously described protocol with some modifications [9].

The methylated and unmethylated MS-PCR condition for *Notch 2* is as follows: PCR was conducted in a total reaction volume of 20 µl containing 60 ng of bisulfite treated DNA, 10 picomole of each primer, 1.5mM $MgCl_2$, 200 µM each deoxyribonucleotide triphosphate (dNTP), 1.0-unit Hot start *Taq* DNA polymerase and 1X PCR buffer (Qiagen, Germany). The product was amplified in a PCR thermal cycler under the following conditions: Initial denaturation at 95˚C for 10 minutes, followed by 35 cycles each, denaturation at 95˚C for 30 seconds, annealing at 58˚C for 30seconds, and elongation at 72˚C for 30 seconds, followed by a final extension at 72˚C for 7 minutes. The methylated and unmethylated MS-PCR

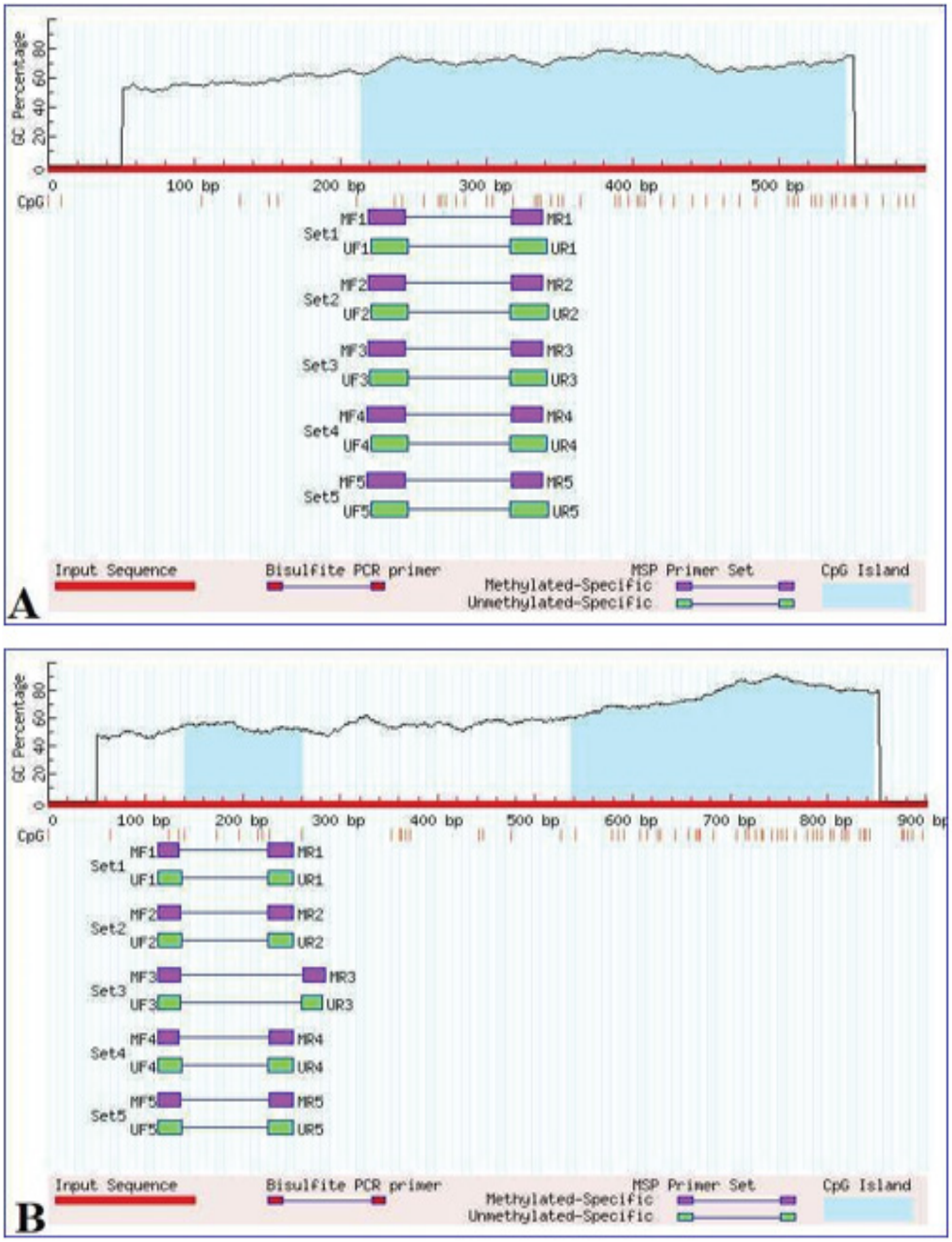

Figure 1: (A) Showing CpG Promoter island of Notch2 (B) CpG promoter island of Notch3 predicted using bioinformatics toll.

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condition for *Notch 3* is as follows: PCR was conducted in a total reaction volume of 20 µl containing 60 ng of bisulfite treated DNA, 10 picomole of each primer, 2.5mM $MgCl_2$, 300 µM each deoxyribonucleotide triphosphate (dNTP), 1.0-unit Hot start *Taq* DNA polymerase and 1X PCR buffer (Qiagen, Germany). The product was amplified in a PCR thermal cycler under the following conditions: Initial denaturation at 95˚C for 08 minutes, followed by 35 cycles each, denaturation at 95˚C for 30 seconds, annealing at 55˚C for 45seconds, and elongation at 72˚C for 30 seconds, followed by a final extension at 72˚C for 5 minutes. Nuclease free water was used in place of bisulfite treated DNA which serves as a negative control and Universal Methylated Human DNA Standard (Zymo research- D5011, USA) was used as a positive control. The amplified PCR product for methylated and unmethylated was checked on 2% agarose gel containing 0.5µg/ml Ethidium bromide and visualized by Gel Doc Imaging system (Biorad, USA).

RNA Extraction and cDNA Synthesis

Total RNA was extracted from the stored tumor and normal tissue sample using Trizol reagent (Thermo Fisher Scientific, USA) according to previously established protocol with some modification [21]. On-column DNase treatment was performed using RNase free DNaseI enzyme (Invitrogen USA). RNA purity was checked using NanoDrop spectrophotometer (Thermo-scientific, New York) and quantified by Qubit 4 fluorometer (Invitrogen, USA). The quality of RNA was checked on 1.2% agarose gel. On the basis of RNA quality samples were selected for integrity checkup. RNA integrity number (RIN) was determined by RNA 6000 Nano kit (Cat No #5067-1511) using Bioanalyzer 2100 (Agilent Technologies Inc., USA) (Figure 2).

cDNA Synthesis: Reverse transcription reactions were performed to obtain cDNA using Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). The total volume of the reaction was 20 µl and containing 500 ng of total RNA template, 5Xreaction buffer; 15 pmol random primer, 20U/µl RiboLock RNase Inhibitor, 10 mM dNTP Mix and 20U/µl Revert Aid Reverse transcriptase. The mixture was prepared in a PCR tube and reaction was performed in thermocycler under the following condition; 5 minutes at 25˚C followed by 60 minutes at 42˚C and reaction was stop by heating at 70˚C for 5 minutes.

Quantitative Real-Time PCR (qRT-PCR)

Quantitative real-time PCR were performed in triplicate for *Notch 2, Notch 3 and Hes1* separately in the final reaction volume of 10 µl containing 5µl of 2X maxima SYBR Green/ ROX qPCR master mix, 0.2 µl of 10 Pico moles of each primer set (forward and reverse), 2 µl of diluted (1:5X) cDNA and 2.6µl nuclease free water. Reverse transcriptase minus (RT-) negative control and no template negative (NTC) was used to access the genomic DNA contamination of the RNA sample and reagent contamination respectively. Amplification was performed in a 96 well plate in CFX Real Time Detection System (Bio-Rad), initial denaturation at 95˚C for 2 minutes, followed by 40 cycles of 95˚C for 15 seconds and 60˚C for 30 seconds. Reaction specificity was checked followed by qPCR using the melting curve analysis (Figure 3). The expression

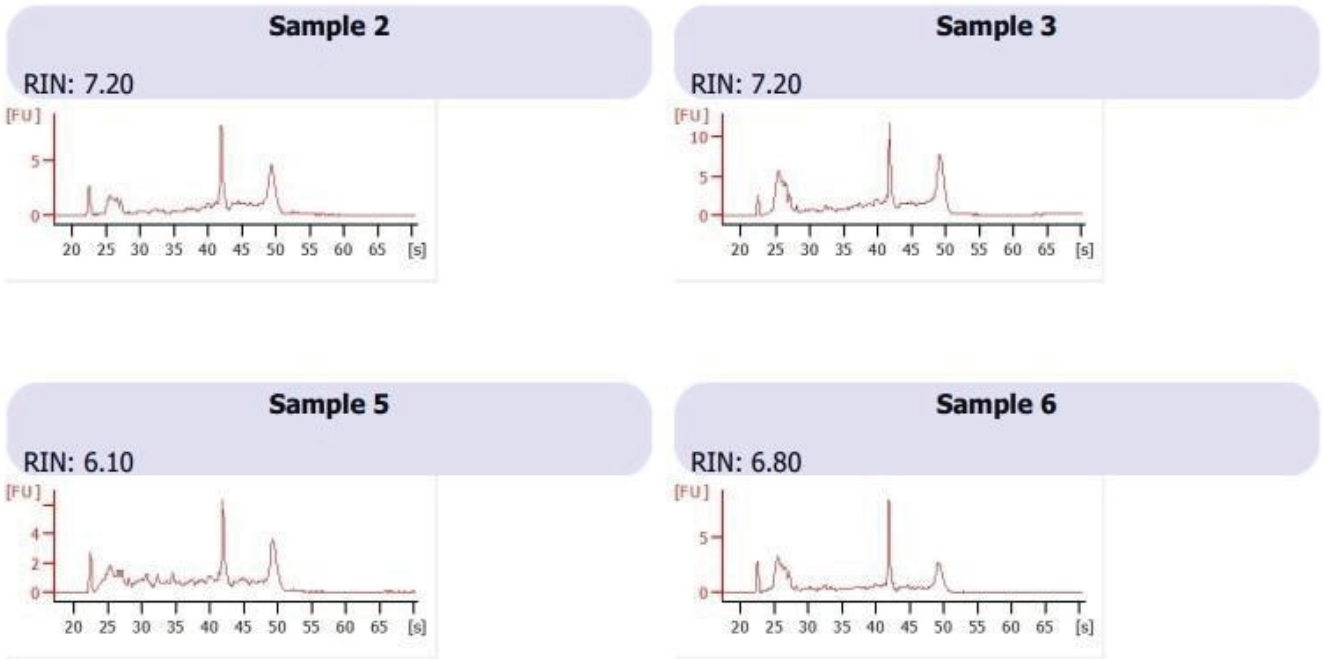

Figure 2: Bioanalyzer electropherogram of RNA samples, representing RNA integrity number (RIN).

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of each was normalized by subtracting the Ct value of the housekeeping gene GAPDH from the Ct value of the tar-get gene. The fold increase, relative to the control, was obtained by using the formula $2^{-\Delta\Delta Ct}$. The sequences of the primers for *Notch 2, Notch 3,* and GAPDH are shown in (Table 1).

Result

Our results suggest that *Notch 2 and Notch 3* could have tumor progression roles in CRC. To analyze the methylation of *Notch 2 & 3* and expression of *Notch 2, 3 and Hes1*, we conducted MS-PCR and Real time PCR respectively.

Notch2 and Notch3 Show Hypomethylation in CRC Patients

We performed methylation specific PCR to analyze the epigenetic changes in CRC tissue. Both methylation and unmethylation patterns in the promoter region were seen in both genes. However, the majority of tumor tissue showed hypomethylated patterns i.e.52/72 (72.22%), and 54/72 (75%) in *Notch 2 and Notch 3* respectively compared to normal (Table 2). Representative agarose gel images of methylation and unmethylation of *Notch2 and Notch3* genes are illustrated in figure 4. Further we correlated hypomethylation patterns of tumor tissue with clinico-pathological characteristics of CRC patients. *Notch 2* hypomethylation showed significant association with advanced TNM stage (p= 0.001), high tumor depth (p=0.003) and presence of lymph node metastasis (p=0.036) (Table 3). The hypomethylation in *Notch3* showed significant correlation with tumor site i.e. rectal tumor (p=0.041), higher TNM stage (p=0.003) and presence of lymph node metastasis (p=0.012) (Table 4).

Upregulated *Notch2 and Notch3* is associated with Advance Disease Stage and Higher Tumor Grade

We evaluated the mRNA level of *Notch 2, Notch 3* and target gene *Hes1* in 72 tumor and adjacent normal tissue using quantitative real-time PCR. We observed that 49 (68.05%) patients, 51(70.84%) patients and 53(73.61%) patients showed over expression in *Notch 2, Notch 3 and Hes1* gene respectively with a fold change increase of 3.37, 5.43 and 3.52 respectively (Figure 5 and 6). In addition, we correlated the mRNA expression with various clinicopathological characteristics. *Notch 2* upregulation was significantly associated with higher tumor stage (p =<0.001 and presence of lymph node metastasis (p=<0.009) (Table 5) whereas *Notch 3* upregulation was significantly associated with higher tumor depth (p=<0.021), presence of lymph node metastasis (p=0.005), advance tumor stage (p=0.007) and high-grade tumors (p=0.013) (Table 6). The overexpression of Hes1 showed significant correlation tumor depth, advance tumor stage and presence of lymph node metastasis (Table 7). We noticed that fold change in overexpression showed increase with worsening of tumor differentiation (p= 0.018), advancing tumor stage (p= <0.001) and presence of LN metastasis (p=0.001), lymphovascular invasion (p=<0.001), perineural invasion (p= 0.014) in *Notch 2* (Table 8). In notch 3, the fold change of expression showed association with tumor grade (p= 0.021), tumor depth (p=<0.001), advance disease stage (p= <0.001), presence of LN metastasis (p=0.001), lymphovascular invasion (p=<0.001) and metastatic disease (Table 9). Similar to *Notch 3, Hes1* also showed higher fold change of expression with tumor depth, advance stage, lymph node metastasis and distant metastasis (Table 10).

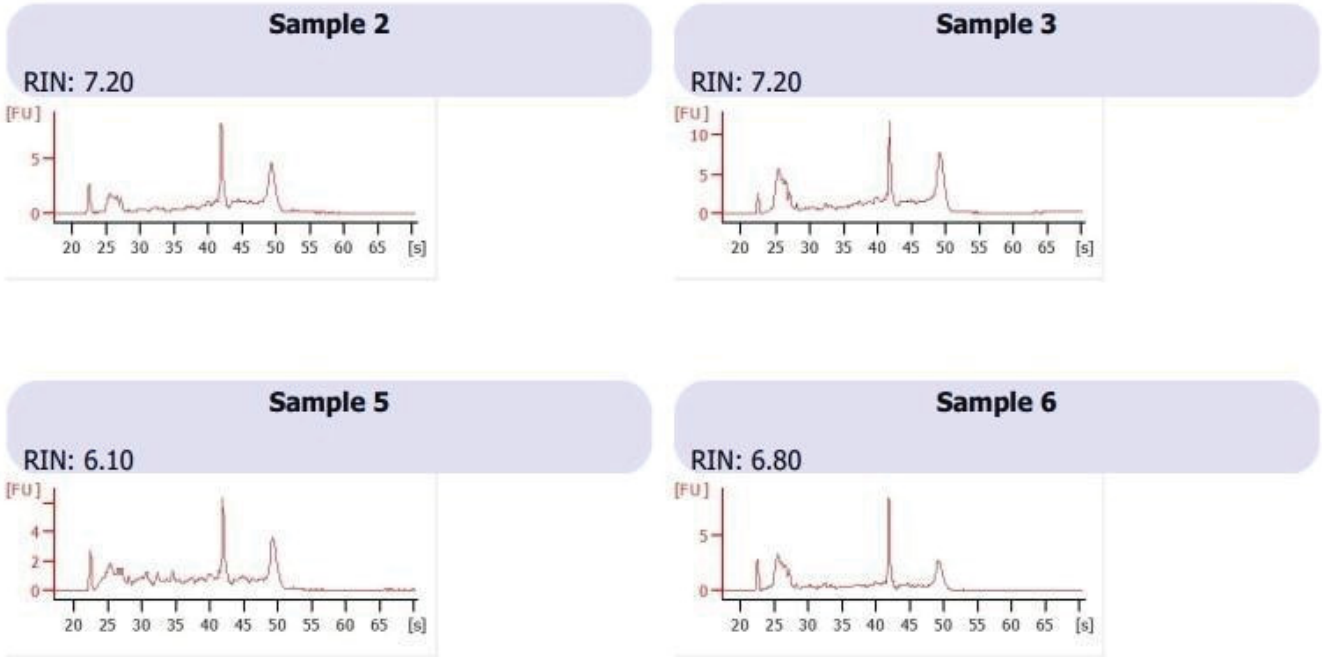

Figure 2: Bioanalyzer electropherogram of RNA samples, representing RNA integrity number (RIN).

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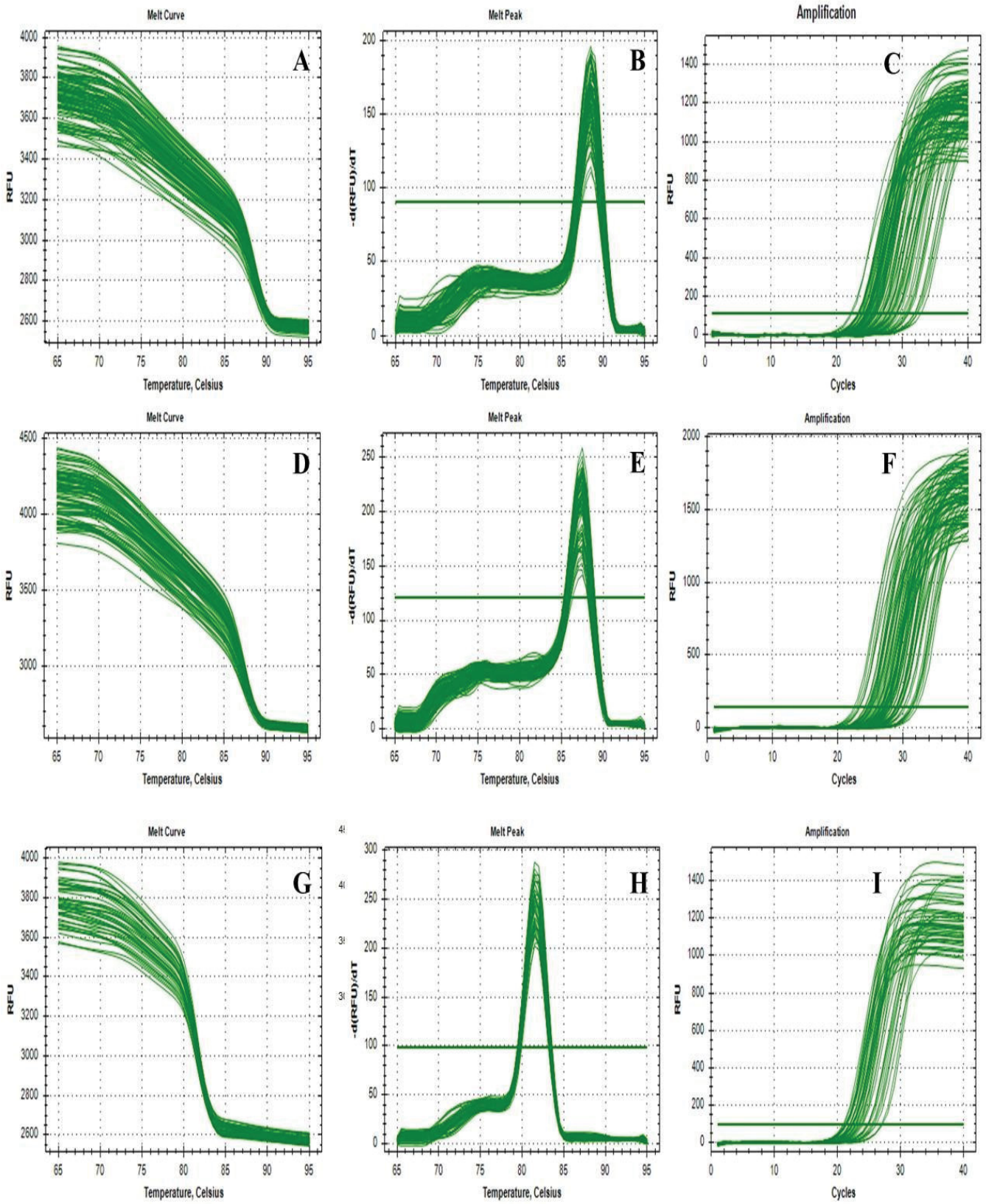

Figure 3: A schematic image of quantitative real-time PCR indicating melt curve a, d, g; melt peak b, e, h and amplification curve in *Notch2, Notch3 and Hes1* gene respectively.

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Table 1: Primer used in quantitative PCR (qPCR) and Methylation Specific PCR (MS-PCR), m- Methylation, um- unmethylation, * Real-Time PCR.

Gene Name	Forward	Reverse	Annealing temp in ˚C	Product length in bp
Notch2*	GTGTTGACTTCTGCTCTCTC	AGTTGGACCTTCTCACTCA	56	200
Notch3*	AGGCTTCACAGGAACCTA	GCTGGTCCACGCATTT	57	200
Hes1*	TCAACACGACACCGGATAAAC	GCCGCGAGCTATCTTTCTTCA	59	153
GAPDH*	AGCGAGATCCCTCCAAA	TTGAGGCTGTTGTCATACT	59	200
Notch2_m	TTTGTATTGGTTAAGTTAGCGAGTC	GCGCGAAAAAATCTACTACGA	58	120
Notch2_um	TGTATTGGTTAAGTTAGTGAGTTGT	TCCACACAAAAAAATCTACTACAAA	58	121
Notch3_m	TTGGGATTATAGGTCGGAGTTATC	ACCGAACACCTCTAAAACCG	55	208
Notch3_um	TTGGGATTATAGGTTGGAGTTATTG	CCAAACACCTCTAAAACCAAA	55	207

Table 2: Methylation status of Notch2 and Notch 3 in normal and tumor CRC tissue.

Tissue type	Notch2 methylation status (n=72)		χ2	*p- value*	Notch3 methylation status (n=72)		χ2	*p- value*
	Hypo Methylation	Hyper Methylation			Hypo Methylation	Hyper Methylation		
Normal	21	51			23	49		
Tumor	52	20	26.70	<0.001	54	18	26.82	<0.001

Table 3: Clinicopathological correlation of *Notch2* methylation in CRC patients.

Clinico-pathologic parameters	Notch2 Methylation (n= 72)		*P* value
	Hypermethylation (n = 20) (27.78%)	Hypomethylation (n = 52) (72.22%)	
Age (years) *≤ 40* *> 40*	 04 16	 16 36	0.558
Gender *Male* *Female*	 10 10	 36 16	0.107
Site *Colon* *Rectum*	 15 05	 34 18	0.173
Grade of differentiation *Well* *Moderate* *Poor*	 01 16 03	 10 29 13	0.141
Tumor Depth *T1* *T2* *T3* *T4*	 02 09 07 02	 07 05 29 11	0.003
Lymph node Metastasis *Positive* *Negative*	 04 16	 24 28	0.036
Metastasis *Yes* *No*	 02 18	 03 49	0.613

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TNM stage *I* *II* *III* *IV*	 04 14 02 00	 06 14 29 03	0.001
Lymphovascular Invasion *Positive* *Negative*	 03 17	 11 41	0.744
Perineural Invasion *Positive* *Negative*	 02 18	 06 46	0.851
Addiction *Yes* *No*	 05 15	 19 33	0.352
Family History *Yes* *No*	 01 19	 03 49	0.693
mRNA Expression *Up-regualte* *Down-regulate*	 08 12	 41 11	0.004

Table 4: Clinicopathological correlation of *Notch3* methylation in CRC patient.

Clinico-pathologic parameters	**Notch3 Methylation (n= 72)**		***P* value**
	Hypermethylation (n = 18) (25%)	**Hypomethylation (n = 54) (75%)**	
Age (years) *≤ 40* *> 40*	 05 13	 15 39	0.627
Gender *Male* *Female*	 09 09	 37 17	0.129
Site *Colon* *Rectum*	 15 03	 31 23	0.041
Grade of differentiation *Well* *Moderate* *Poor*	 04 10 04	 07 35 12	0.64
Tumor Depth *T1* *T2* *T3* *T4*	 03 03 12 00	 04 10 27 13	0.100
Lymph node Metastasis *Positive* *Negative*	 02 16	 26 28	0.012
Metastasis *Yes* *No*	 0 18	 5 49	0.226
TNM stage *I* *II* *III* *IV*	 03 13 02 00	 07 15 29 03	0.003
Lymphovascular Invasion *Positive* *Negative*	 02 16	 12 42	0.253
Perineural Invasion *Positive* *Negative*	 02 16	 06 48	1.00

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Addiction			
Yes	08	16	0.192
No	10	38	
Family History			
Yes	00	04	0.307
No	18	50	
mRNA Expression			
Up-regulate	05	46	<0.001
Down-regulate	13	08	

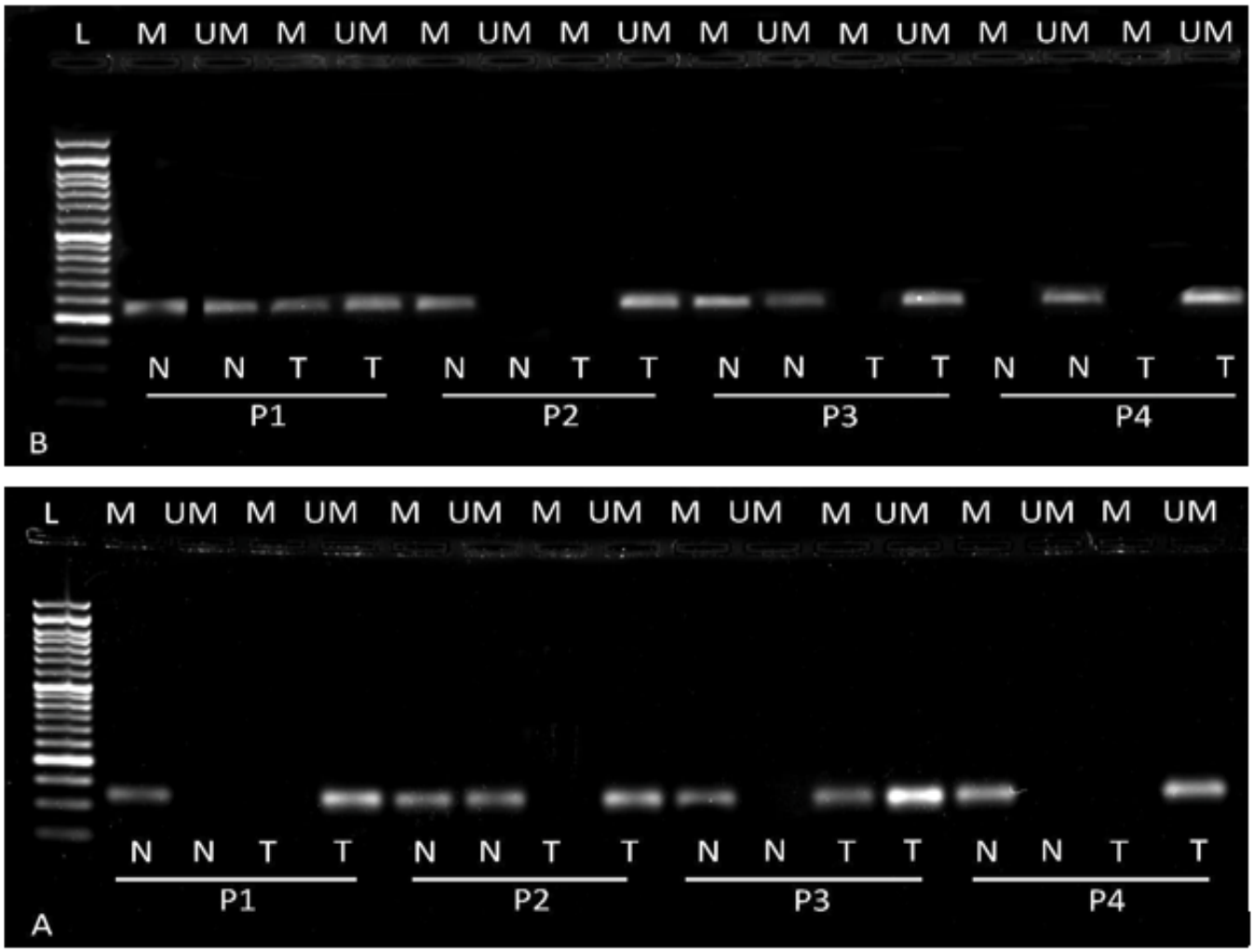

Figure 4: Representative Agarose gel image of Methylation Specific PCR (A) Notch2 (B) Notch3 in CRC patients. L- 50bp DNA ladder; N-normal tissue; T- tumor tissue; M- methylated DNA; UM- unmethylated; P- patient.

Correlation between Notch2 and Notch3 Overexpression with Hypomethylation in CRC Patients

The relationship between overexpression and hypomethylation of *Notch 2 and Notch 3* genes was examined. The overexpression of *Notch 2* showed a significant correlation with hypomethylation in tumor tissue (p=0.002) (Table 4). Similarly, Notch 3 overexpression was associated with presence of hypomethylation in tumor tissue (p=0.001) (Table 5).

Association between Notch2 and Notch3 and Target Gene Hes1

We found very strong positive correlation between *Notch 2 and Notch 3* upregulation and *Hes1* overexpression. We found that *Hes1* was upregulated in 78% of cases where *Notch 2* was overexpressed, and similar outcomes were seen in 79% of cases where *Notch 3* was overexpressed (Table 7). Overall this finding suggested that both Notch signaling and Hes1 may be involved in CRC disease progression.

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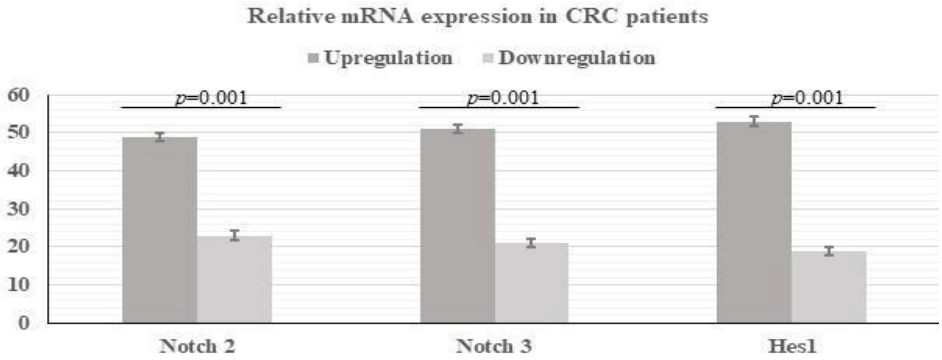

Table 5: Clinicopathological correlation of *Notch 2* mRNA expression in CRC patient.

Figure 5: Relative mRNA expression of *Notch2, Notch3* and Hes1 in CRC patients.

Clinico-pathologic parameters	Notch2 mRNA expression (n= 72)		*P* value
	Upregulate (n = 49) (68.05%)	downregulate (n = 23) (31.95%)	
Age (years)			
≤ 40	14	06	1.00
> 40	35	17	
Gender			
Male	33	13	0.435
Female	16	10	
Site			
Colon	31	15	1.00
Rectum	18	08	
Grade of differentiation			
Well	06	05	0.326
Moderate	30	15	
Poor	13	03	
Tumor Depth			
T1	04	03	0.354
T2	07	06	
T3	27	12	
T4	11	02	
Lymph node Metastasis			
Positive	24	04	0.009
Negative	25	19	
Metastasis			
Yes	04	01	1.00
No	45	22	
TNM stage			
I	05	05	<0.001
II	13	15	
III	28	03	
IV	03	00	
Lymphovascular Invasion			
Positive	12	02	0.114
Negative	37	21	
Perineural Invasion			
Positive	06	02	0.655
Negative	43	21	
Addiction			
Yes	18	06	0.372
No	31	17	
Family History			
Yes	02	02	0.425
No	47	21	
Methylation			
Hypermethylation	08	12	0.002
Hypomethylation	41	11	

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Table 6: Clinicopathological correlation of *Notch3* mRNA expression in CRC patient.

Clinico-pathologic parameters	Notch 3 mRNA expression (n= 72)		*P* value
	Upregulate (n = 51) (70.84%)	Downregulate (n = 21) (29.16%)	
Age (years)			
≤ 40	16	04	0.390
> 40	35	17	
Gender			
Male	33	13	0.514
Female	18	08	
Site			
Colon	31	15	0.433
Rectum	20	06	
Grade of differentiation			
Well	04	07	0.013
Moderate	33	12	
Poor	14	02	
Tumor Depth			
T1	02	05	0.021
T2	08	05	
T3	29	10	
T4	12	01	
Lymph node Metastasis			
Positive	25	03	0.005
Negative	26	18	
Metastasis			
Yes	05	00	0.312
No	46	21	
TNM stage			
I	03	07	0.007
II	19	09	
III	26	05	
IV	03	00	
Lymphovascular Invasion			
Positive	12	02	0.209
Negative	39	19	
Perineural Invasion			
Positive	07	01	0.423
Negative	44	20	
Addiction			
Yes	17	07	0.603
No	34	14	
Family History			
Yes	03	01	1.00
No	48	20	
Methylation			
Hypermethylation	05	13	<0.001
Hypomethylation	46	08	

Citation: Abhay Kumar Sharma, Nimisha, Apurva, Arun Kumar, Asgar Ali, Sundeep Singh Saluja, Birendra Prasad. Elevated Expression of Notch 2 & Notch 3 is associated with Disease Progression in Colorectal Cancer. International Journal of Applied Biology and Pharmaceutical Technology 13 (2022): 033-050.

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Table 7: Clinicopathological correlation of *Hes1* mRNA expression in CRC patient.

Clinico-pathologic parameters	Hes1 mRNA expression (n= 72)		*P* value
	Upregulate (n = 53) (73.61%)	Downregulate (n = 19) (26.38%)	
Age (years)			
≤ 40	16	04	0.328
> 40	37	15	
Gender			
Male	34	12	0.575
Female	19	07	
Site			
Colon	33	13	0.425
Rectum	20	06	
Grade of differentiation			
Well	06	03	0.269
Moderate	34	11	
Poor	13	05	
Tumor Depth			
T1	04	03	<0.001
T2	04	09	
T3	32	07	
T4	13	00	
Lymph node Metastasis			
Positive	24	04	0.054
Negative	29	15	
Metastasis			
Yes	04	01	0.603
No	49	18	
TNM stage			
I	04	06	0.034
II	20	08	
III	26	05	
IV	03	00	
Lymphovascular Invasion			
Positive	12	02	0.214
Negative	41	17	
Perineural Invasion			
Positive	08	00	0.074
Negative	45	19	
Addiction			
Yes	18	06	0.544
No	35	13	
Family History			
Yes	03	01	0.717
No	50	18	
Notch 2 mRNA Expression			
Up-regulate	37	12	0.397
Down-regulate	16	07	
Notch 3 mRNA Expression			
Up-regulate	40	11	0.126
Down-regulate	13	08	

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Table 8: Clinicopathological correlation of *Notch2* mRNA fold change in CRC patient.

Clinico-pathologic parameters	n = 72 (%)	Notch 2 mRNA expression mean ± SD	*P* value
Age (years)			
≤ 40	20 (0.28)	2.44±0.80	0.032
> 40	52 (0.72)	2.04±0.27	
Gender			
Male	46 (0.63)	2.61±0.39	0.409
Female	26 (0.37)	2.10±0.39	
Site			
Colon	46 (0.63)	2.32±0.37	0.617
Rectum	26 (0.37)	2.32±0.46	
Grade of differentiation			
Well	11 (15.28)	1.84±0.56	0.018
Moderate	45 (62.50)	2.03±0.24	
Poor	16 (22.22)	3.96±1.00	
Tumor Depth			
T1	07 (0.09)	1.62±0.55	0.111
T2	13 (0.18)	1.13±0.71	
T3	39 (0.54)	2.80±1.84	
T4	13 (0.19)	3.03±1.88	
Lymph node Metastasis			
Positive	28 (38.88)	3.84±0.61	<0.001
Negative	44 (61.12)	1.53±0.18	
Metastasis			
Yes	05 (0.07)	2.92±1.17	0.649
No	67 (0.93)	2.39±0.30	
TNM stage			
I	10 (0.13)	1.25±0.35	<0.001
II	28 (0.39)	1.10±0.15	
III	31 (0.43)	3.85±0.52	
IV	03 (0.45)	4.02±1.73	
Lymphovascular Invasion			
Positive	14 (0.20)	4.42±1.01	<0.001
Negative	58 (0.80)	1.95±0.23	
Perineural Invasion			
Positive	08 (0.11)	4.43±1.69	0.014
Negative	64 (0.89)	2.18±0.24	
Addiction			
Yes	24 (0.33)	2.45±0.46	0.961
No	48 (0.67)	2.42±0.37	
Family History			
Yes	04 (0.06)	3.16±1.95	0.547
No	68 (0.94)	2.38±0.29	

Table 9: Clinicopathological correlation of *Notch3* mRNA fold change in CRC patient.

Clinico-pathologic parameters	n = 72 (%)	Notch2 mRNA expression mean ± SD	*P* value
Age (years)			
≤ 40	20 (0.28)	3.16±0.70	0.297
> 40	52 (0.72)	4.23±0.57	
Gender			
Male	46 (0.63)	4.18±0.60	0.480
Female	26 (0.37)	3.50±0.69	
Site			
Colon	46 (0.63)	3.51±0.57	0.221
Rectum	26 (0.37)	4.68±0.74	

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Grade of differentiation			
Well	11 (15.28)	1.54±1.54	0.021
Moderate	45 (62.50)	3.94±0.54	
Poor	16 (22.22)	5.61±1.20	
Tumor Depth			
T1	07 (0.10)	0.73±0.25	<0.001
T2	13 (0.18)	2.62±0.67	
T3	39 (0.54)	3.82±1.59	
T4	13 (0.18)	7.29±1.19	
Lymph node Metastasis			
Positive	28 (38.88)	5.49±0.78	0.006
Negative	44 (61.12)	2.94±0.51	
Metastasis			
Yes	05 (0.07)	8.92±1.68	0.002
No	67 (0.93)	3.56±0.44	
TNM stage			
I	10 (0.13)	1.48±0.66	<0.001
II	28 (0.39)	2.91±0.57	
III	31 (0.43)	4.91±0.72	
IV	03 (0.05)	11.50±0.77	
Lymphovascular Invasion			
Positive	14 (0.20)	6.38±1.39	0.008
Negative	58 (0.80)	3.34±0.43	
Perineural Invasion			
Positive	08 (0.11)	5.31±1.73	0.289
Negative	64 (0.89)	3.76±0.46	
Addiction			
Yes	24 (0.33)	3.82±0.80	0.869
No	48 (0.67)	3.98±0.56	
Family History			
Yes	04 (0.05)	4.36±2.12	0.821
No	68 (0.95)	3.91±0.47	

Table 10: Clinicopathological correlation of *Hes1* mRNA fold change in CRC patient.

Clinico-pathologic parameters	n = 72 (%)	Hes1 mRNA expression mean ± SD	*P* value
Age (years)			
≤ 40	20 (0.28)	2.78±0.49	0.473
> 40	52 (0.72)	2.40±0.26	
Gender			
Male	46 (0.63)	2.45±0.28	0.748
Female	26 (0.37)	2.61±0.37	
Site			
Colon	46 (0.63)	2.31±0.24	0.276
Rectum	26 (0.37)	2.85±0.48	
Grade of differentiation			
Well	11 (15.28)	2.49±0.88	0.979
Moderate	45 (62.50)	4.48±0.27	
Poor	16 (22.22)	2.60±0.44	
Tumor Depth			
T1	07 (0.10)	1.57±0.42	0.003
T2	13 (0.18)	1.09±0.48	
T3	39 (0.54)	2.77±0.34	
T4	13 (0.18)	3.65±0.50	
Lymph node Metastasis			
Positive	28 (38.88)	3.24±0.44	0.013
Negative	44 (61.12)	2.04±0.24	
Metastasis			
Yes	05 (0.07)	4.06±1.68	0.073
No	67 (0.93)	2.39±0.44	

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TNM stage			
I	10 (0.13)	1.29±0.32	<0.001
II	28 (0.39)	1.90±0.24	
III	31 (0.43)	3.14±0.41	
IV	03 (0.05)	5.69±0.47	
Lymphovascular Invasion			
Positive	14 (0.20)	3.12±0.53	0.205
Negative	58 (0.80)	2.36±0.26	
Perineural Invasion			
Positive	08 (0.11)	3.54±0.74	0.122
Negative	64 (0.89)	2.38±0.24	
Addiction			
Yes	24 (0.33)	2.18±0.32	0.337
No	48 (0.67)	2.67±0.31	
Family History			
Yes	04 (0.05)	2.66±1.09	0.879
No	68 (0.95)	2.50±0.24	

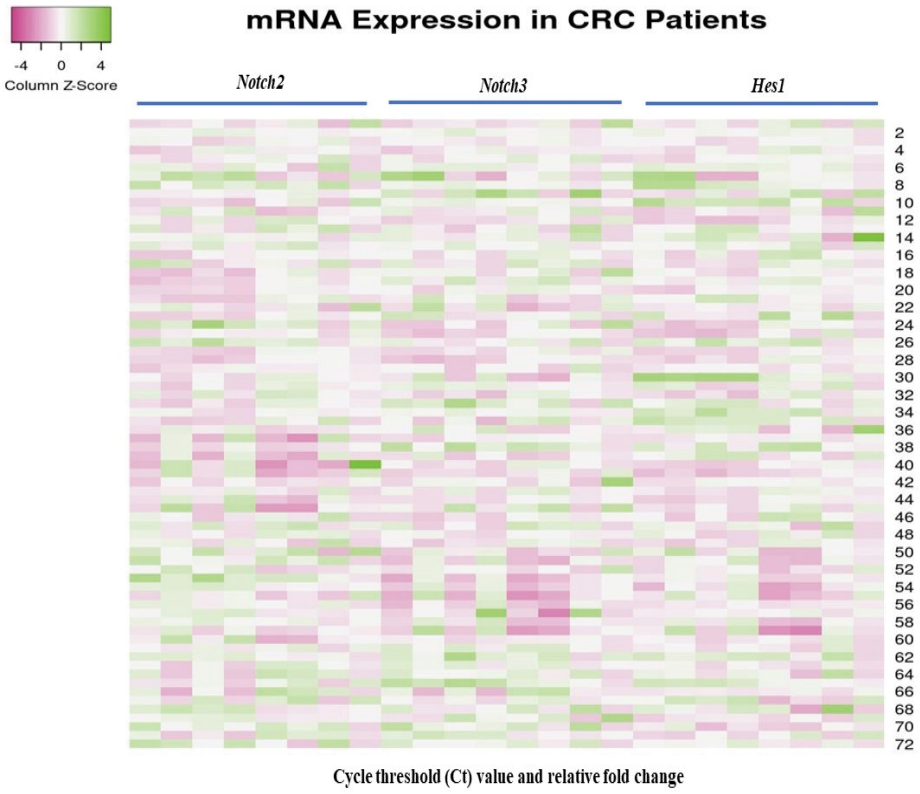

Figure 6: Heat map plot of relative mRNA expression and fold change; *Notch 2, Notch 3 and Hes1* in CRC patients analyzed using online tool heat mapper.

Discussion

Despite both clinical and molecular advancements in colorectal cancer, the incidence and disease progression has not shown much change. Recent colorectal staging system has included molecular markers for prognosis and use of targeted therapy, still more biomarkers are need to be explored. In colonic crypts, Notch signaling pathway plays major role in maintaining the balance between cell proliferations and cell fate determination by regulating colon stem cell behaviour and differentiation [22,23]. Each Notch receptor has a distinct function at cellular level. *Notch2* and *Notch3* are key members of the Notch family that regulate cellular functions. The functions of these depend upon the type of tumor, suggesting that they may not always play the same roles in different malignancies. [24–26]. Notch3 signaling regulates cellular activities during the progression of cancer and maintains the stemness of cancer stem cells (CSCs) [27]. Another key characteristic of Notch3 is the development of tumor

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resistance to several chemotherapy agents such as platinum, doxorubicin, gemcitabine, and EGFR tyrosine kinase inhibitors (TKIs).[27–29]. We analyzed the methylation pattern of *Notch 2 &3* and expression of *Notch 2, Notch 3* and *Hes1* in 72 colorectal cancer patients. Consequently, we correlated the findings with clinicopathological characteristics to decipher its role in colorectal cancer. Epigenetic instability plays a crucial role in the development of cancer by the blocking of cell proliferation and cell cycle arrest [30,31]. Recent studies have focused on DNA methylation that plays a vital role in the regulation of gene expression by the blocking of transcription factor binding sites on the promoter region. We report hypomethylation of *Notch 2 and 3* genes in tumor tissue in 72% and 75% patients respectively. The hypomethylation showed significant correlation with clinical parameters including higher tumor depth, advance stage and presence of lymph node metastasis, suggesting their association in tumor progression. The hypomethylation also showed significant correlation (Notch 2; p=0.004, Notch 3; p=0.001) with expression in approx. 90 % cases suggesting it could be one of the regulatory mechanisms controlling notch 2 & 3 gene expression in colorectal cancer. Previous reports suggest distinct miRNA such as 23b and 133a regulate notch 2 gene at translation level in gastric cancer [12]. miRNA-23b's capacity to directly bind to the *Notch2* mRNA and prevent its translation made it possible to stop tumor development by restoring its expression [12] As a substitute for the activation of the γ -secretase complex, miRNA-133a can target and inhibit the translation of presenilin 1, inhibiting the release of NICD and obstructing the pro-oncogenic function of Notch signaling. Our results indicate that mRNA expression was upregulated in 68% and 70.84% in *Notch 2 and Notch3* genes respectively in CRC patients. Notch 2 overexpression results in abnormal proliferation and dedifferentiation resulting in tumor development in gastric cancer. Similar findings are observed in our study in colorectal cancer where Notch 2 expression was higher in poorly differentiated tumors and advance stages. Though the notch 2 overexpression showed significant correlation with perineural and lymphovascular invasion, however it should be further evaluated as the number of patients are less. Moreover, previous study by Chu et al showed *Notch 2* function is opposite to Notch 1 and inversely associated with differentiation and tumor stage. Similar role of *Notch 2* as oncogene is reported in gastric cancer (71.4%) laryngeal squamous cell carcinoma (87.3%) and medulloblastoma (74.4%) [13,19,32]. In gastric cancer *Notch 2* intracellular domain (N2ICD) activation has been shown to encourage the increased expression of cyclooxygenase-2 (COX-2) and promotes the epithelial-mesenchymal transition (EMT) in tumor cells [33]. The increased expression of *Notch 3* showed significant association with tumor grade, tumor depth, TNM stage and presence of lymph node metastasis. Unlike *Notch 2, Notch 3* expression was significantly higher in fold change in patients with metastatic disease compared to non-metastatic disease (8.92±1.68 vs 3.56±0.44 p = 0.002). Similar results were reported in the CRC tissue and mice model indicating that increased *Notch 3* expression was associated with distant metastasis and poor prognosis [34,35]. In ovarian cancer, *Notch 3* expression was linked to tumor grade, lymph node, distant metastasis, and clinical stage [36–38]. However, there is disagreement concerning the function of *Notch 3* in breast tumors, although some researchers reported that *Notch 3* encourages tumor aggressiveness by triggering EMT, other researchers showed that Notch 3 actually inhibits it. Moreover, it has been reported that the pathogenesis of HCC may be aided by the activation of Notch 3 signaling, which reduces the Wnt/-catenin signaling and enhances the expression of the protein Nanog linked to stemness. [39]. Similar association of *Notch3 and Wnt* pathway should be looked at in CRC to further explore its effect on CSCs. *Hes1* is a well-known Notch signaling target gene. It is a novel bHLH transcriptional repressor which is overexpressed in colorectal cancer [21,40]. It can be activated when cleaved fragments of Notch intracellular domain enters into the nucleus, connect with DNA-binding protein, and transform DNA-binding protein into *Hes1* activator. Therefore, *Hes1* expression has been considered as a marker for Notch activation. The Notch signaling regulates *Hes1* in several cancers including colorectal, oral squamous cell carcinoma and pancreatic [38,41,42]. We found a substantial relationship between activation of *Notch 2 and 3 with Hes1* expression. Hes1 showed high expression in 73.61% cases. This finding suggests the activation of *Notch 2 and 3* could have resulted in raised Hes1 as notch target gene in colorectal cancer. This could be one of the mechanisms by which the Notch pathway can result in tumor progression in colorectal cancer. Candy et al found overexpression of *Hes1* in 60% patients in colorectal tumors. They found its value in predicting survival with chemotherapy in combination with other Notch induced transcription factors HEY1 and SOX 9 [43]. However, Reedijk et al analyzed Notch activation using *Hes1* expression as surrogate marker [44]. Although the expression was observed in all patients, raised expression was seen in 31% tumor tissue compared to normal and did not correlate with survival. Our results suggest that Notch signaling activation may occur due to aberrant hypomethylation of Notch promoter and its activation can result in overexpression of *Hes1*. Both *Notch 2 & 3* played a role as an oncogene in CRC. These findings support to explore use of Notch receptor inhibitors in reducing tumor burden and improving survival.

Limitation and Future Prospective

Although our study has pointed towards the role of Notch signaling pathway in CRC management; however, the sample size and availability of metastatic samples were the major limitations. Moreover, the activation of Notch signaling depends on the interaction between Notch receptor and ligands which regulate the downstream target gene.

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Therefore, it is important to analyze all the members of Notch family to decode their actual role in disease progression. Further, the cell line-based study should be targeted which can aid in novel therapeutic approaches.

Conclusion

Our results suggest that the promoter hypomethylation influences the mRNA upregulation of *Notch 2 and Notch 3* in CRC. The aberrant methylation pattern and dysregulated gene expression of *Notch 2 and Notch 3* was found to be associated with advanced stage, nodal metastasis and increased tumor depth in CRC. Our additional finding indicates that the gene expression of *Hes1* transcription factor may be regulated by *Notch 2 and 3* receptor genes Therefore, it can be stated that the DNA methylation of *Notch 2 and Notch 3* promoters may have great potential in clinical applications. It may also prove as useful indicator for the development and progression of CRC. But before coming to a definitive conclusion this research needs further exploration to replicate our significant findings.

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Conflicts of Interest

No competing interests exist.

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