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Original Article

Aberrant promoter methylation of *NOTCH1* and *NOTCH3* and its association with cervical cancer risk factors in North Indian population

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Abstract: Cervical cancer is the fourth most common type of cancer in women worldwide, and associated mortality is highest in developing countries like India. Limited studies are available on the role of NOTCH signaling pathway and promoter methylation in cervical cancer. In the current study, we investigated the promoter methylation status of NOTCH receptor genes (mainly *NOTCH1, NOTCH2,* and *NOTCH3*) and its correlation with gene expression, clinicopathological factors, and prognosis of cervical cancer. A total cohort of 110 cervical cancer patients of North Indian origin was enrolled in the study. From 28 of these patients, biopsies from adjacent non-cancerous tissue were available to serve as healthy controls. Promoter methylation status and mRNA expression level of *NOTCH1, NOTCH2,* and *NOTCH3* were determined by methylation-specific PCR (MSP) and real-time quantitative (RT-qPCR), respectively. *NOTCH1* and *NOTCH3* promoters were methylated in 92% ($P<0.0001$), and 61% ($P<0.001$) of the cervical cancer biopsies. We did not observe a statistically significant change in the promoter methylation level of *NOTCH2*. Further, *NOTCH1, NOTCH2,* and *NOTCH3* were down-regulated in cervical cancer biopsies, but the differential expression of only *NOTCH1* was found statistically significant. The promoter methylation levels of all three genes also showed a statistically significant association with clinicopathological factors and HPV infection (Type 16 and 18) but we did not observe a statistically significant relationship between their methylation status and gene expression. Overall our results provide evidence of the altered methylation and expression status of *NOTCH1* and *NOTCH3* receptor genes in cervical cancer. This study of NOTCH gene promoter methylation may provide a new perspective for early screening and diagnosis of cervical cancer.

Keywords: Cervical cancer, NOTCH signaling pathway, promoter methylation, gene expression, HPV16 and 18, clinicopathological factors

Introduction

According to the World Health Organization, 570,000 new cases of cervical cancer are diagnosed annually, representing 6.6% of all female cancers. Approximately 90% of cervical cancer-related deaths occur in middle and low-income nations, and India shares a significant burden of this high rate of mortality [1]. More than 122,844 women in India get diagnosed with cervical cancer, out of which 67,477 dies of the disease every year [2]. In India, the incidence rate of cervical cancer is close to 2% (1 in 53), which is about twice (1 in 100) as high as in more developed regions of the world [3]. Role of the human papillomavirus (HPV) in the pathogenesis of cervical cancer is well established as a persistent infection by high-risk serotypes of HPV (mainly type 16 and 18) confers a higher risk of cervical cancer development. However, infection by HPV alone is not sufficient as additional genetic and epigenetic alterations in the affected women are required for progression from precancerous disease to invasive cancer [4, 5].

NOTCH pathway is an evolutionarily conserved intercellular signaling pathway that regulates interactions between physically adjacent cells, patterning, cell fate decisions, cell proliferation,

and growth [6]. NOTCH pathway gets triggered by the interaction of NOTCH receptors (NOTCH1, NOTCH2, NOTCH3, and NOTCH4) with NOTCH ligands (*DLL1, DLL4, JAG1,* and *JAG2*) [7]. Each NOTCH receptor is encoded by a different NOTCH gene (*NOTCH1, NOTCH2, NOTCH3,* and *NOTCH4*). Abnormal activation of the NOTCH pathway plays a crucial role in tumor development and progression in different malignant cancers [8]. In a variety of cell types, the NOTCH signaling impedes apoptosis and activates proliferation, and in some cell types, it inhibits tumor growth. Thus, depending on the cell type, NOTCH genes can act as oncogenes or as tumor-repressor genes. Further, this dual nature of NOTCH genes is context-dependent and varies in different malignancies [9].

NOTCH genes act as an oncogene in acute T-cell lymphoblastic leukemia (T-ALL), breast cancer, gastric cancer, and lung cancer [10-13]. Over-expression of NOTCH3 was observed in human lung cancer cell line, and its inhibition using targeted therapy decreased the proliferation of cells in vitro [13]. In small cell lung cancer, bladder cancer and in low-grade gliomas, NOTCH signaling has the tumor-suppressive role [14-16]. In small lung cancer cell lines, over expression of NOTCH1 and NOTCH2 arrests the growth of cells [14]. Inhibition of over-expressed NOTCH receptors (NOTCH3 and NOTCH4) and NOTCH ligand (JAG1) in pancreatic cancer tissue resulted in tumor suppression while over-expression of NOTCH2 was reported in pancreatic lesions and loss of expression of *NOTCH2* inhibited progression of pancreatic cancer [17]. In embryonal brain tumor, *NOTCH2* promotes the progression of the tumor, whereas, *NOTCH1* inhibit the tumor growth [18].

NOTCH signaling pathway also plays a crucial role in the development and progression of cervical cancer [19, 20]. Previous studies showed that *NOTCH1* is down-regulated in cervical cancer tissues in comparison to healthy cervix tissues while *NOTCH3* was found to be over-expressed in cervical cancer tissues, promoting proliferation and survival of tumor cells [21, 22]. The mechanism leading to deregulation of NOTCH expression is not known yet. Recently, a trend of increased tumorogenesis was found with abnormal methylation pattern during the multistage carcinogenesis of cervical cancer [23]. Hypermethylation of CpG islands in promoter regions is usually coupled with decreased gene expression and is very frequently found in specific tumors [4]. The study of methylation status of the NOTCH genes may provide a potential mechanism through which they are deregulated in cervical cancer. The abnormal methylation status of the NOTCH pathway can be used as a biomarker for early diagnosis and can provide legitimate targets for cervical cancer therapy.

We explored the promoter methylation status and gene expression of the NOTCH receptor genes (*NOTCH1, NOTCH2,* and *NOTCH3*) in cervical cancer patients to evaluate their association in order to gain insight into the epigenetic regulation of NOTCH signaling in cervical cancer. Further, we have studied the correlation of promoter methylation with different clinicopathological factors and with HPV infection (HPV16 and 18) in order to understand its potential use in the early diagnosis of cervical cancer. To our knowledge, no such studies on promoter methylation of the NOTCH receptor genes have been reported yet in cervical cancer.

Material and methods

Study design

The present study was conducted on cervical biopsies collected from 110 married women with cervical abnormalities (94 cervical cancer biopsies, 16 chronic cervicitis biopsies) from Department of Obstetrics and Gynecology, Pandit Bhagwat Dayal Sharma Health University, Haryana, India, from September 2016 to August 2018. From 28 of these patients, biopsies from adjacent healthy tissue were available, that served us as healthy control biopsies. Written informed consent was obtained from all enrolled cases, and relevant clinicopathological parameters were collected after their clinical examination. Pathologists carried out the histopathological analysis of collected biopsies and classified them to various grades of cervical tumors. All samples were processed in a Biosafety Level II laminar flow hood in the Department of Genetics, Maharishi Dayanand University, Rohtak, Haryana, India. Ethical approval for sample collection was obtained from the institutional human ethical committee (IHEC) with Number IHEC/2016/80-13.06.16.

Table 1. Primer for Real-Time PCR (RT-PCR) and Methylation specific PCR (MSP)

Name	Forward Primer	Reverse Primer	Annealing temperature (°C)	Product size (bp)
MSP Primers				
NOTCH1 (M)	TGGTTTTTGAAAATTTTTAAACGA	ATAAAACTCAAAATCGAAATACGCT	61.5	263
NOTCH1 (UM)	TTGGTTTTTGAAAATTTTTAAATGA	AATAAAACTCAAAATCAAAATACACT	61.5	265
NOTCH2 (M)	TTTGTATTGGTTAAGTTAGCGAGTC	GCGCGAAAAAATCTACTACGA	55.5	120
NOTCH2 (UM)	TGTATTGGTTAAGTTAGTGAGTTGT	TCCACACAAAAAAATCTACTACAAA	55.5	121
NOTCH3 (M)	TTGGGATTATAGGTCGGAGTTATC	ACCGAACACCTCTAAAACCG	60.7	208
NOTCH3 (UM)	TTGGGATTATAGGTTGGAGTTATTG	CCAAACACCTCTAAAACCAAA	63	207
Real Time Primers				
NOTCH1	TCGACGATTGTCCAGGAA	GACACACACGCAGTTGTAG	58	110
NOTCH2	GTGTTGACTTCTGCTCTCTC	AGTTGGACCTTCTCACTCA	58	110
NOTCH3	AGGCTTCACAGGAACCTA	GCTGGTCCACGCATTT	58	110
GAPDH	AGCGAGATCCCTCCAAA	CTTGAGGCTGTTGTCATACT	58	110

(M-Methylated DNA specific primer, UM-Unmethylated DNA specific primer, °C: Celcius; bp: Base pair).

Sample collection and genomic DNA isolation

Fresh cervical biopsies were collected and preserved in RNA later solution (Invitrogen; Thermo Fisher Scientific, Inc.) and stored at -80°C for further processing. Genomic DNA Isolation from tissue samples was performed by using STE buffer -100 mM NaCl, 10 mM Tris, and 1 mM EDTA (Sisco Research Laboratories, India). Briefly, tissues were crushed in Liquid N_2 and re-suspended in STE buffer and then incubated with 100 µg of proteinase K (Invitrogen; Thermo Fisher Scientific, Inc.) at 55°C for 16 h. DNA was extracted using phenol, chloroform, and isoamyl-alcohol mixture (25:24:1) and further precipitated by using ethanol. Extracted DNA samples were stored at -20°C for future use. The concentration and purity of genomic DNA were determined by Nanodrop Spectrophotometer (mySPEC, Sigma-SVi). Samples with an OD_{260}/OD_{280} ratio between 1.8-2.0 were included in the study. Additionally, the integrity of genomic DNA was also confirmed by 0.8% agarose gel electrophoresis.

Bisulfite conversion

300 ng of freshly isolated genomic DNA was modified by Sodium bisulfite using MethylCode™ Bisulfite Conversion Kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) as per manufacturer's instructions. Bisulfite conversion of DNA was carried out at 98°C for 10 minutes followed by 2.5 hours incubation at 64°C during which unmethylated cytosine was converted into uracil completely. Bisulfite converted DNA was stored at -20°C until further usage.

Primers for methylation specific PCR (MSP) and real time quantitative PCR (qPCR)

For detection of promoter methylation, primers specific for either methylated or unmethylated promoter region (**Table 1**) were designed using Methprimer online tool. Sequences for NOTCH gene promoter regions were retrieved from the Eukaryotic Promoter Database. Primers for qPCR were designed using OligoAnalyzer 3.1 software. PCR were performed using Hot Start *Taq*-DNA-polymerase (New England Biolabs, USA), 0.2 mM dNTPs, 1 uM primers and by following cycling protocol: initial denaturation at 98°C for 10 minutes followed by 40 cycles of 95°C for 30 seconds, 55°C to 63°C for 30 seconds and 72°C for 30 seconds; and a final extension at 72°C for 10 minutes. The PCR products were visualized after electrophoresis on ethidium-bromide stained 3% agarose gel.

RNA extraction and reverse-transcription real-time quantitative PCR

Since all biopsies were not sufficient for the isolation of both DNA and RNA, therefore RNA was isolated from only 70 cervical cancer biopsies and 18 adjacent normal biopsies. RNA extraction was performed by Trizol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) using manufacturer guidelines with slight modifications as per requirement, and the concentration was determined by Nanodrop Spectrophotometer

Table 2. Socio-demographic and clinico-pathological features of the cervical cancer study population

Sociodemographic & Clinicopathological Features	Number of Patients (N)	Percentage (%)
Age		
<55 years	42	38.18
≥55 years	68	61.82
Age at time of marriage		
≤18	53	48.18
>18	57	51.81
Rural	86	78.18
Urban	24	21.81
Number of births		
≥3	60	54.54
<3	50	45.45
Number of abortions		
0-2	101	91.81
≤3	9	8.18
Menopause		
Pre	43	39.09
Post	67	60.90
Menstrual Hygiene		
Poor	72	65.45
Good	38	34.54
Histological grading		
Cervicitis	16	14.54
Squamous Cell Carcinoma	90	81.81
Adenocarcinoma	2	1.81
Adenosquamous carcinoma	2	1.81
Differentiation degree		
Unknown	46	48.93
Poor	8	8.51
Moderate	46	48.93
Well	10	10.64

(mySPEC, Sigma-SVi). 100 ng of RNA was used for cDNA synthesis, which was carried out using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific# K1622). Quantitative real-time PCR was performed using Maxima SYBR Green/ROX qPCR Master Mix (Applied Biosystem; Thermo Fisher Scientific, Inc.) in Step One Plus Real-Time PCR system (Applied Biosystem; Thermo Fisher Scientific, Inc.). Cycling conditions of 10 minutes at 94°C, 40 cycles of 95°C for 15 seconds and 58°C for 45 seconds, 1 minute at 72°C were used for PCR. Expression of the NOTCH genes was calculated relative to GAPDH expression as $\Delta Ct=Ct_{GAPDH}-Ct_{NOTCH}$. Larger ΔCt values represent higher relative NOTCH expression.

Statistical analysis

GraphPad Prism 8 software (GraphPad, California, USA) was used to perform the statistical analysis. Odds ratio (95% confidence interval) and Fisher's exact test were used to compare the promoter methylation status of NOTCH genes in cancer and normal biopsies and to evaluate the correlation between the promoter methylation pattern and the clinicopathological factors and HPV infection. Gene expression levels of Notch genes in cervical cancer and healthy tissues were compared using Student's t-test, and the correlation between promoter methylation and gene expression was studied using the Pearson correlation coefficient and t-test. A *p*-value of ≤0.05 was considered statistically significant.

Results

Demographic and clinical features of cervical cancer biopsies

Sociodemographic features and histological grading of all cervical cancer cases included in the study are shown in **Table 2**. Mean age and age at marriage were 55.3 and 15.95 with an age range of 28-82 years and 14-21 years respectively. Most of the cases (84.4%) were illiterate/just literate and belonged to the rural background (60.3%). Bleeding after menopause and bleeding during intercourse were the main symptoms in cervical cancer cases. Differentiation degree was unknown for 41.8% cancer biopsies, while 41.8% biopsies showed moderate differentiation, 9.1% showed well differentiation and 7.3% showed poor differentiation.

Methylation profile of NOTCH1, NOTCH2 and NOTCH3 in cervical cancer and in healthy biopsies by methylation-specific PCR (MSP)

In order to determine promoter methylation pattern of NOTCH receptor genes, we analyzed the methylation status of *NOTCH1, NOTCH2,* and *NOTCH3* in cervical cancer biopsies and healthy biopsies (**Figure 1**). *NOTCH1* and *NOTCH3* promoters were found to be methylated in 92% and 61% of cervical cancer biopsies respectively while NOTCH2 promoter showed methylation in only 36% biopsies. In the case of

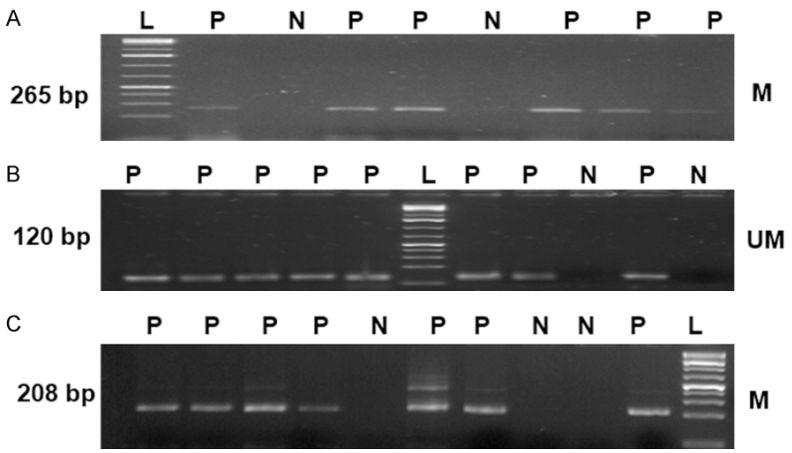

Figure 1. Representative images of Methylation specific PCR (MSP) analysis for NOTCH1, NOTCH2 and NOTCH3 in cervical cancer biopsies. NOTCH1 and NOTCH3 showed maximum methylation whereas Notch2 showed maximum un-methylation (A) NOTCH1 MSP. L: 100 bp DNA ladder; P: samples positive for methylation, N: samples negative for methylation (B) NOTCH2 MSP. L: 100 bp DNA ladder; P: samples positive for un-methylated DNA, N: samples negative for un-methylated DNA (C) NOTCH3 MSP. L: 100 bp DNA ladder; P: samples positive for methylation, N: samples negative for methylation (M: methylation-specific PCR and UM: unmethylation-specific PCR).

normal biopsies, *NOTCH2* promoter was un-methylated in 82% while *NOTCH1* and *NOTCH3* were un-methylated in 64% and 61% of the biopsies respectively. Also, *NOTCH3* promoter was positive for both methylated and unmethylated DNA in 25% of normal biopsies which can be a result of partial methylation. When we compared the methylation status of individual Notch genes in normal biopsies with that of cervical cancer biopsies, a significant difference in *NOTCH1* (P=0.0001) and *NOTCH3* (P=0.001) promoter methylation was observed. Promoters of Notch1 and Notch3 gene are majorly methylated in cervical cancer biopsies and un-methylated in the healthy biopsies. We did not observe a statistically significant difference of *NOTCH2* promoter methylation between normal and cervical biopsies in our cohort of samples (**Table 3**).

Relationship of promoter methylation of NOTCH1, NOTCH2 and NOTCH3 with patient age, menopause status and disease status

The relationship between the promoter methylation status of NOTCH genes with cervical cancer risk factors (age, menopause status, histological grading, and differentiation degree) was analyzed by Fisher's exact test and Odds ratio calculation (95% Confidence interval) (**Table 4**). When methylation frequency of *NOTCH1* and *NOTCH2* promoter was compared with individual age, a significant difference was observed between females above the age of 55 years and below 55 years. Similar observations were observed for menopause status of the women. Postmenopausal women showed significant association with promoter methylation status of *NOTCH2* (P=0.004) and *NOTCH3* (P=0.01). We did not observe a significant relationship between differentiation degree (well, moderate and poor differentiation) and methylation frequency of NOTCH genes. 93% of *NOTCH1* methylated biopsies belonged to cervicitis and squamous cell carcinoma grades showing a significant association (P=0.03) amongst these. Women of age ≥55 years had a higher frequency of *NOTCH1* and *NOTCH3* promoter methylation while *NOTCH2* promoter was mainly unmethylated (84%) in this age group. Women having cervicitis and squamous cell carcinoma and exhibiting poor differentiation degree had *NOTCH1* promoter majorly methylated. This correlation study points towards the possible involvement of *NOTCH1* promoter methylation status as early diagnosis and severity marker in cervical cancer.

Detection of HPV infection in cervical cancer samples

Out of 110 cervical cancer biopsies, 97% were positive for HPV DNA. Amongst those, 84% were HPV16 positive, and 73% samples were positive for HPV18 (**Table 5**), as reported in our previous study [24]. 56% samples were found positive for both HPV16 and HPV18 infection. The HPV infection has a high prevalence in cervical cancer cases, and serotype 16 infection is more frequent than HPV18 in cervical cancer patients belonging to the northern part of India. HPV16 infection exhibits statistically significant positive association with methylation status of Notch1 (P=0.003) and Notch3 (P=0.011) gene

Table 3. Promoter methylation status of *NOTCH1, NOTCH2* and *NOTCH3* in normal and cancerous biopsies

Gene	Biopsies	M	UM	M+UM	OR	95% CI	*p*-value
NOTCH1	Healthy (N=28)	10 (36%)	18 (64%)	0	0.05	0.02-0.14	<0.0001
	Cancer (N=110)	101 (92%)	9 (8%)	0			
NOTCH2	Healthy (N=28)	5 (18%)	23 (82%)	0	0.4	0.16-1.1	0.11
	Cancer (N=110)	39 (36%)	71 (65%)	0			
NOTCH3	Healthy (N=28)	4 (14%)	17 (61%)	7 (25%)	0.15	0.05-0.46	0.001*
	Cancer (N=110)	67 (61%)	43 (39%)	0			
Gene	Biopsies	M	UM	M+UM	OR	95% CI	*p*-value
NOTCH1	Healthy (N=28)	10 (36%)	18 (64%)	0	0.05	0.02-0.14	<0.0001
	Cancer (N=110)	101 (92%)	9 (8%)	0			
NOTCH2	Healthy (N=28)	5 (18%)	23 (82%)	0	0.4	0.16-1.1	0.11
	Cancer (N=110)	39 (36%)	71 (65%)	0			
NOTCH3	Healthy (N=28)	4 (14%)	17 (61%)	7 (25%)	0.15	0.05-0.46	0.001*
	Cancer (N=110)	67 (61%)	43 (39%)	0			

(M: Methylated; UM: Un-methylated; OR: Odds ratio; CI: Confidence of interval; N: Number; *Significant at P≤0.05).

while HPV18 infection showed a significant positive association with only methylation status of Notch1 (P=0.012) gene. We did not observe a strong positive association between HPV infection and methylation of Notch2 gene.

Correlation of HPV infection with NOTCH1, NOTCH2 and NOTCH3 promoter methylation in cervical cancer

In order to study the possible effect of HPV infection (Type 16 and 18) on modifications of the NOTCH receptor genes, we checked correlation of HPV infection with promoter methylation status of NOTCH genes. We found that the HPV16 infection is significantly associated with *NOTCH1* (P=0.003), *NOTCH2* (P=0.04) and *NOTCH3* (P=0.011) promoter methylation, while HPV18 infection is significantly associated with only *NOTCH1* (P=0.0122) and *NOTCH2* (P=0.0001) promoter methylation. No significant association was observed between cases having co-infection by type 16 and 18 and promoter methylation of NOTCH genes (**Table 5**). The *NOTCH1* promoter methylation increased with an increase in HPV16 and HPV18 infection while, the methylation level of *NOTCH3* increased with an increase in HPV16 infection only. On the other hand, number of cases with *NOTCH2* promoter methylation decreased with increase in HPV16 and HPV18 infection. Our data indicates that an infection by HPV (type 16 and 18) might modulate the promoter methylation status of NOTCH genes in cervical cancer cases which coincides with earlier studies showing that HPV E6 protein regulates the activity of Notch pathway in cervical cancer [25]. Further, *Notch*1 promoter methylation status in addition to HPV16 infection status can be a strong marker for early diagnosis and screening of cervical cancer.

Expression of NOTCH1, NOTCH2 and NOTCH3 in cervical cancer biopsies by real-time PCR

The biological significance of promoter methylation of NOTCH receptor genes in cervical cancer was evaluated by expression analysis of NOTCH genes (*NOTCH1, NOTCH2,* and *NOTCH3*) in cervical cancer biopsies and normal biopsies and significance was determined using Student's t-test. Gene expression data of NOTCH receptor genes (*NOTCH1, NOTCH2,* and *NOTCH3*) in cervical cancer biopsies and healthy biopsies is shown in **Figure 2**. In the current study, only NOTCH1 gene expression showed statistically significant difference in cervical cancer biopsies and normal biopsies. We did not observe a statistically significant difference in expression of NOTCH2 and NOTCH3 (**Table 6**).

In our study, *NOTCH1* showed significant down-regulation (P=0.006) in cervical cancer tissues in comparison to healthy tissues, indicating its potential role as a tumor suppressor gene. Both *NOTCH2* and *NOTCH3* showed substantial down-regulation in cervical cancer tissues in

Table 4. Relationship between promoter hypermethylation of *NOTCH1, NOTCH2* and *NOTCH3* and clinicopathological features

Clinico-pathological Factors	Total (110)	Methylation status of *NOTCH1*		OR (95% CI)	*p*-value	Methylation status of *NOTCH2*		OR (95% CI)	*p*-value	Methylation status of *NOTCH3*		OR (95% CI)	*p*-value
		M (101)	UM (9)			M (39)	UM (71)			M (67)	UM (43)		
Age													
<55 years	42	35 (83%)	7 (17%)	0.15 (.03-0.8)	0.03*	28 (67%)	14 (33%)	11 (4.3-24)	0.0001*	21 (50%)	21 (50%)	0.48 (0.22-1.1)	0.07
≥55 years	68	66 (97%)	2 (3%)			11 (16%)	57 (84%)			46 (68%)	22 (32%)		
Menopause													
Pre	43	40 (93%)	3 (7%)	1.3 (.3-5.5)	1	8 (19%)	35 (81%)	0.3 (0.1-0.6)	0.004*	20 (47%)	23 (53%)	0.37 (0.17-0.82)	0.016*
Post	67	61 (91%)	6 (9%)			31 (46%)	36 (54%)			47 (70%)	20 (30%)		
Histological grading													
Cervicitis & SCC	106	99 (93%)	7 (7%)	14 (1.9-95)	0.03*	37 (35%)	69 (65%)	0.54 (0.07-4)	0.61	64 (60%)	42 (40%)	0.5 (.04-3.5)	0.9
ADC & ASC	4	2 (50%)	2 (50%)			2 (50%)	2 (50%)			3 (75%)	1 (25%)		
Differentiation degree*													
Poor	8	8 (100%)	0	Infinity	0.05*	3 (38%)	5 (63%)	1.3 (0.3-5.8)	1	4 (50)	4 (50)	1.7 (0.4-6.1)	0.7
Well & Moderate	56	36 (64%)	20 (36%)			18 (32%)	38 (68%)			21 (38)	35 (63)		

(M: Methylated; UM: Unmethylated; SCC: Squamous Cell Carcinoma; ADC: Adenocarcinoma; ASC: Adenosquamous Carcinoma; OR: Odd ratio; CI: Confidence of interval; *Significant at P≤0.05).

Table 5. Correlation of HPV infection with *NOTCH1, NOTCH2* and *NOTCH3* methylation in cervical cancer

HPV Type	N (110)	Methylation status of *NOTCH1*		OR (95% CI)	*p*-value	Methylation status of *NOTCH2*		OR (95% CI)	*p*-value	Methylation status of *NOTCH3*		OR (95% CI)	*p*-value
		M (101)	UM (9)			M (39)	UM (71)			M (67)	UM (43)		
HPV16+	90	87 (97%)	3 (3%)	15.6 (3.4-59)	0.003*	28 (31%)	62 (69%)	0.4 (0.14-1)	0.04*	60 (67%)	30 (33%)	3.7 (1.3-9.4)	0.011*
HPV18+	78	73 (94%)	5 (6%)	2.1 (0.6-7.7)	0.012*	18 (23%)	60 (77%)	0.2 (.06-0.4)	0.001*	44 (56%)	34 (44%)	0.5 (0.2-1.1)	0.19
HPV16+ & 18+	60	58 (97%)	2 (3%)	4.7 (0.9-23)	0.07	18 (30%)	42 (70%)	0.6 (0.3-1.3)	0.2	40 (67%)	20 (33%)	1.7 (0.8-3.8)	0.2

(N: Number; HPV: Human Papillomavirus; M: Methylated; UM: Unmethylated; OR: Odd Ratio; CI: Confidence of interval; *Significant at P≤0.05).

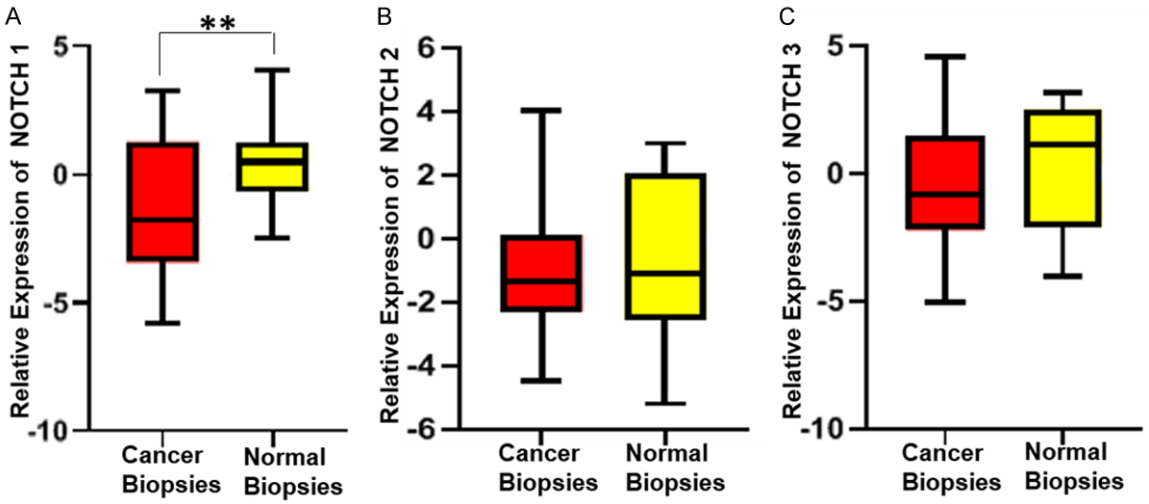

Figure2. NOTCH1 promoter hypermethylation significantly silenced gene expression. Expression analysis of (A) NOTCH1, (B) NOTCH2 and (C) NOTCH3 genes in 70 cervical cancer and 18 normal tissues by Real-Time Quantitative Reverse Transcription PCR. Relative expression level of each gene was determined using ΔCt=Ct(GAPDH)-Ct(NOTCH) and expression level of GAPDH was used as internal control. Statistical significance determined by t-test and *P<0.05 indicates a significant difference between cervical cancer and normal tissues.

Table 6. Comparison of gene expression levels (ΔCt) of *NOTCH1, NOTCH2* and *NOTCH3* in cervical cancer patients and in healthy biopsies

Gene	Healthy biopsies (N=18) Mean± SD	Cancer biopsies (N=70) Mean± SD	(95% CI)	*p*-value
NOTCH1	0.41±1.5	-1.32±2.47	0.50-2.95	0.006*
NOTCH2	-0.62±2.41	-1.17±1.9	-0.52-1.6	0.31
NOTCH3	0.12±2.52	-0.57±2.2	-0.51-1.9	0.26

N: Number; SD: Standard Deviation; CI: Confidence of interval; *Significant at P≤0.05.

comparison to normal tissues, but the difference was not found to be statistically significant. Non-significant change in gene expression of *NOTCH2* and *NOTCH3* might be a consequence of the high degree of variation in their gene expression levels amongst our cohort of samples. Since individual Notch receptors play different cell type-specific role; therefore, *NOTCH1* might be the major modulator of the Notch signaling pathway in cervical cancer. Since we did observe a statistically significant difference in expression of Notch2 and Notch3 genes therefore correlation studies between methylation and gene expression were performed only for Notch1 gene.

Correlation of gene expression with NOTCH1 promoter methylation in cervical cancer

In order to study the potential effect of *NOTCH1* promoter methylation status on corresponding gene expression, we segregated cervical cancer and normal cervix biopsies into two subgroups one in which *NOTCH1* is methylated and second subgroup had un-methylated *NOTCH1* promoter. *NOTCH1* mRNA expression in the methylated subgroup of cervical cancer is -1.46±2.49 and un-methylated subgroup is -0.96±2.29 while that in methylated subgroup of normal cervix biopsies is 0.11±0.81 and the un-methylated subgroup is 0.67±1.76. In cervical cancer biopsies and normal cervix biopsies, mRNA expression of *NOTCH1* was considerably high in un-methylated subgroup than in methylated subgroup, which indicates a negative correlation between promoter methylation status and *NOTCH1* expression. However, Pearson correlation analysis and unpaired t-test showed an only small negative correlation, which might be because of the small sample number in our study. Further studies need to be done with more samples to get a more conclusive result about the promoter methylation mediated expression of *NOTCH1* in cervical cancer.

Discussion

Cervical cancer is one of the common malignancies in women worldwide, accounting for 17% of cancer deaths among women aged between 30-69 years. Notably, in developing

countries like India, the prevalence and mortality rates of cervical cancer remain high due to lack of screening and proper medical facilities for treatment [2]. Age, multiple pregnancies, abortions, and use of oral contraceptives play an essential role in the development of cervical cancer [26]. In our study, including women of North India, we found that cervical cancer is more prevalent among women older than 55 years, in post-menopause stages, belonging to rural areas and who had poor menstrual hygiene. Our data indicate a great need for comprehensive screening of women living in rural parts of India and the need for programs to elaborate the importance of menstrual hygiene in reducing risks of cervical cancer.

Abnormal promoter methylation in cancer cells has achieved increasing recognition as an essential mechanism for silencing of the tumor suppressor genes and activation of oncogenes, which eventually contribute to carcinogenesis [27]. An altered methylation pattern represents a stable and frequent change during cervical carcinogenesis [28]. Furthermore, contrary to genetic alterations, DNA methylation is reversible that makes it useful for therapy purposes, efforts are required to find and validate these findings using population-based studies. Also, increasing pieces of evidence have shown that HPV infection hinders cellular DNA methylation machinery and during HPV16/18 infection, cellular DNA experiences epigenetic changes such as abnormal DNA methylation [29, 30]. Therefore, the knowledge of methylation changes of signaling pathways genes in association with viral infection is vital for targeted therapies.

Epidemiological data and molecular observations have revealed that persistent infection by high-risk serotypes of HPV is a crucial risk factor for cervical cancer development [26]. Previous studies have shown that the incidence of infection by HPV type 16 and 18 in cervical cancer is very high as compared to other HPV types. The available literature shows that the prevalence of HPV infection in women in different parts of India ranges from 9 to 99% [31-34]. In our study; the prevalence of HPV infection was much higher (97.3%) with a significant infection of HPV16 (84.1%) and HPV18 (72.9%), and in many cases, we observed co-infection by HPV16 and 18. We analyzed the association of HPV16 and 18 infections with the methylation status of the NOTCH genes (*NOTCH1, NOTCH2,* and *NOTCH3*) and found that infection by HPV16 and 18 is significantly associated with methylation status of NOTCH genes in cervical cancer biopsies.

The NOTCH signaling pathway is an evolutionarily conserved pathway involved in cell fate determination, cell proliferation and apoptosis. Aberrant NOTCH signaling is associated with several diseases, including cancer, where the state of the cell gets altered, and the cell adopts a proliferative stage [8]. In cervical cancer, both oncogenic as well as tumor-suppressive properties of Notch signaling have been described. In some studies, it was found that NOTCH overexpression has tumor-suppressive effects, like inhibition of growth and activation of the apoptotic pathway [21, 35, 36] while other studies have established that increased Notch expression associated with the development of cervical cancer and contributes to the survival of cancer cells [22, 25, 37]. These studies have reported the deregulation of the NOTCH pathway in cervical cancer, but till now, there are very few studies on the molecular mechanism of NOTCH pathway deregulation. For the first time, to check the plausible epigenetic mechanism of the NOTCH pathway deregulation, we analyzed the promoter methylation status of NOTCH receptor genes (*NOTCH1, NOTCH2*, and *NOTCH3*) in cervical cancer biopsies and normal biopsies and evaluated the effect of promoter methylation on expression of these genes.

NOTCH1 is one of the four receptors which are involved in the NOTCH signaling pathway. Previous studies have shown that *NOTCH1* is shown to exhibits both oncogenic as well as tumor-suppressive functions in different cancers [10, 38, 39]. In cervical cancer, the role of the *NOTCH1* is controversial. In some studies, *NOTCH1* expression was high in cervical cancer while in others it was lower in comparison to the normal cervix; however to best of our knowledge, the methylation status of the *NOTCH1* in cervical cancer is not described in any study previously. An increased understanding of the role of *NOTCH1* methylation in the development of cervical cancer may provide novel approaches into the process of tumorogenesis.

In the present study, *NOTCH1* promoter was methylated in 92% of cervical cancer biopsies and only 36% healthy biopsies (**Table 3**). The relative promoter methylation of *NOTCH1* in cervical cancer biopsies was found to be signifi-

cantly higher (P=0.0001) than normal biopsies. Relationship of age and menopausal status with promoter methylation is evidenced in previous studies showing that an increasing age and menopause status increases the promoter methylation level [40, 41]. In the present study, a significant positive association of *NOTCH1* promoter methylation was observed with age, histological grading differentiation degree, and with HPV16 and HPV18 infection. We report for the first time that there is an increase in *NOTCH1* promoter methylation with the increase in age, severity of the disease, and HPV infection (type 16 and 18). Our observations highlight the importance of studying *NOTCH1* promoter methylation along with HPV screening as an early screening and diagnostic marker for cervical cancer pathogenesis. To further study the molecular implication of change in promoter methylation in cervical cancer biopsies in comparison to healthy biopsies, we looked at the corresponding effect on the expression of these genes. The real-time PCR results showed that *NOTCH1* is significantly down-regulated (P=0.006) in cervical cancer in comparison to the normal cervix. In order to study plausible promoter methylation mediated effect on gene expression, we performed correlation analysis which showed only small negative correlation (Pearson correlation coefficient, r=-0.16) between these in case of *NOTCH1* in cervical cancer. However, we suspect that this might be because of our small sample size, and further investigations need to be performed on this potential regulation mechanism.

The NOTCH2 receptor is another primary regulator of Notch signaling, which plays an essential role in cell fate determination. In the current study, we observed that only 35% of cervical cancer biopsies were methylated and 65% of cervical cancer biopsies were un-methylated (**Table 3**). No statistically significant difference in *NOTCH2* promoter methylation status was seen between cervical cancer biopsies and healthy biopsies. However, we observed a significant positive association of *NOTCH2* promoter methylation with age, post-menopausal stage, and HPV16 and 18 infection. *NOTCH2* promoter methylation showed an inverse relationship with age, menopause status and with HPV infection (type 16 and 18) as the frequency of promoter methylation decreases with increase in age, the menopause status and HPV infection. Relationship of HPV16 and 18 infections with the promoter methylation of *NOTCH2* in cervical cancer patients is reported only in the present study. Furthermore, in the real-time PCR data, the difference in expression was not found statistically significant in cervical cancer and healthy biopsies. In cancer, the role of *NOTCH2* remains controversial as some studies in bladder cancer, medulloblastomas, cervical cancer and in pancreatic cells, have shown that *NOTCH2* function as an oncogene, which advances the tumor progression [15, 17, 18, 37] while an independent study showed that the expression of *NOTCH2* remained unaffected in cervical cancer [21].

Our results showed that *NOTCH3* promoter was methylated in 61% cervical cancer biopsies and only 14% of healthy biopsies. A significant difference of *NOTCH3* promoter methylation was found between normal and cancer biopsies (P=0.001) as shown in **Table 3**. We observed a significant association of *NOTCH3* with menopause status and with HPV16 infection, showing that *NOTCH3* promoter methylation increases with increase in age and HPV16 infection. Previous studies in cervical, ovarian, and pancreatic cancer reported *NOTCH3* as an oncogene, where it helps in the proliferation and survival of tumor cells [22, 25, 42, 43]. In the current study, real-time PCR results did not show a significant difference in *NOTCH3* expression between normal biopsies and in cervical cancer biopsies. Further, studies using more number of biopsies might provide a better understanding of the status of *NOTCH2* and *NOTCH3* in cervical cancer.

The discovery and characterization of epigenetic targets may prove particularly attractive in designing novel strategies for inhibiting cancer development and progression. Here in this study, we report for the first time about the aberrant methylation of NOTCH receptor genes in cervical cancer patients of North India and their positive association with HPV infection (Type 16 and 18) and other risk factors. This study is particularly important because the promoter methylation status of NOTCH genes may provide a new perspective for early diagnosis and treatment of cervical cancer. However, to evaluate the potential benefits of NOTCH-targeted therapy for cervical cancer, additional research is needed to study the effect of altered methylation on the NOTCH signaling pathway in detail.

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Disclosure of conflict of interest

None.

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References

[1] Kaarthigeyan K. Cervical cancer in India and HPV vaccination. Indian J Med Paediatr Oncol 2012; 33: 7-12.

[2] Sreedevi A, Javed R and Dinesh A. Epidemiology of cervical cancer with special focus on India. Int J Womens Health 2015; 7: 405-14.

[3] Institute for Health Metrics and Evaluation. The challenge ahead: progress and setbacks in breast and cervical cancer. Seattle, WA: IHME; 2011.

[4] Costello JF, Frühwald MC, Smiraglia DJ, Rush LJ, Robertson GP, Gao X, Wright FA, Feramisco JD, Peltomäki P, Lang JC, Schuller DE, Yu L, Bloomfield CD, Caligiuri MA, Yates A, Nishikawa R, Su Huang H, Petrelli NJ, Zhang X, O'Dorisio MS, Held WA, Cavenee WK and Plass C. Aberrant CpG-island methylation has non-random and tumour-type-specific patterns. Nat Genet 2000; 24: 132-8.

[5] Weijzen S, Zlobin A, Braid M, Miele L and Kast WM. HPV16 E6 and E7 oncoproteins regulate Notch-1 expression and cooperate to induce transformation. J Cell Physiol 2003; 194: 356-362.

[6] Artavanis-Tsakonas S, Rand MD and Lake RJ. Notch signaling: cell fate control and signal integration in development. Science 1999; 284: 770-776.

[7] Mumm JS and Kopan R. Notch signaling: from the outside in. Dev Biol 2000; 228: 151-165.

[8] Aithal MG and Rajeswari N. Role of Notch signalling pathway in cancer and its association with DNA methylation. J Genet 2013; 92: 667-675.

[9] Miele L, Miao H and Nickoloff BJ. NOTCH signaling as a novel cancer therapeutic target. Curr Cancer Drug Targets 2006; 6: 313-323.

[10] Weng AP, Ferrando AA, Lee W, Morris JP, Silverman LB, Sanchez-Irizarry C, Blacklow SC, Look AT and Aster JC. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. Science 2004; 306: 269-271.

[11] Robinson DR, Kalyana-Sundaram S, Wu YM, Shankar S, Cao X, Ateeq B, Asangani IA, Iyer M, Maher CA, Grasso CS, Lonigro RJ, Quist M, Siddiqui J, Mehra R, Jing X, Giordano TJ, Sabel MS, Kleer CG, Palanisamy N, Natrajan R, Lambros MB, Reis-Filho JS, Kumar-Sinha C and Chinnaiyan AM. Functionally recurrent rearrangements of the MAST kinase and Notch gene families in breast cancer. Nat Med 2011; 17: 1646-51.

[12] Hsu KW, Hsieh RH, Huang KH, Fen-Yau Li A, Chi CW, Wang TY, Tseng MJ, Wu KJ and Yeh TS. Activation of the Notch1/STAT3/Twist signaling axis promotes gastric cancer progression. Carcinogenesis 2012; 33: 1459-1467.

[13] Osanyingbemi-Obidi J, Dobromilskaya I, Illei PB, Hann CL and Rudin CM. Notch signaling contributes to lung cancer clonogenic capacity in vitro but may be circumvented in tumorigenesis in vivo. Mol Cancer Res 2011; 9: 1746-1754.

[14] Sriuranpong V, Borges MW, Ravi RK, Arnold DR, Nelkin BD, Baylin SB and Ball DW. Notch signaling induces cell cycle arrest in small cell lung cancer cells. Cancer Res 2001; 61: 3200-3205.

[15] Hayashi T, Gust KM, Wyatt AW, Goriki A, Jäger W, Awrey S, Li N, Oo HZ, Altamirano-Dimas M, Buttyan R and Fazli L. Not all NOTCH is created equal: the oncogenic role of NOTCH2 in bladder cancer and its implications for targeted therapy. Clin Cancer Res 2016; 22: 2981-2992.

[16] Cancer Genome Atlas Research Network, Brat DJ, Verhaak RG, Aldape KD, Yung WK, Salama SR, Cooper LA, Rheinbay E, Miller CR, Vitucci M, Morozova O, Robertson AG, Noushmehr H, Laird PW, Cherniack AD, Akbani R, Huse JT, Ciriello G, Poisson LM, Barnholtz-Sloan JS, Berger MS, Brennan C, Colen RR, Colman H, Flanders AE, Giannini C, Grifford M, Iavarone A, Jain R, Joseph I, Kim J, Kasaian K, Mikkelsen T, Murray BA, O'Neill BP, Pachter L, Parsons DW, Sougnez C, Sulman EP, Vandenberg SR, Van Meir EG, von Deimling A, Zhang H, Crain D, Lau K, Mallery D, Morris S, Paulauskis J, Penny R, Shelton T, Sherman M, Yena P, Black A, Bowen J, Dicostanzo K, Gastier-Foster J, Leraas KM, Lichtenberg TM, Pierson CR, Ramirez NC, Taylor C, Weaver S, Wise L, Zmuda E, Davidsen T, Demchok JA, Eley G, Ferguson ML, Hutter CM, Mills Shaw KR, Ozenberger BA, Sheth M, Sofia HJ, Tarnuzzer R, Wang Z, Yang L, Zenklusen JC, Ayala B, Baboud J, Chudamani S, Jensen MA, Liu J, Pihl T, Raman R, Wan Y, Wu Y, Ally A, Auman JT, Balasundaram M, Balu S, Baylin SB,

Beroukhim R, Bootwalla MS, Bowlby R, Bristow CA, Brooks D, Butterfield Y, Carlsen R, Carter S, Chin L, Chu A, Chuah E, Cibulskis K, Clarke A, Coetzee SG, Dhalla N, Fennell T, Fisher S, Gabriel S, Getz G, Gibbs R, Guin R, Hadjipanayis A, Hayes DN, Hinoue T, Hoadley K, Holt RA, Hoyle AP, Jefferys SR, Jones S, Jones CD, Kucherlapati R, Lai PH, Lander E, Lee S, Lichtenstein L, Ma Y, Maglinte DT, Mahadeshwar HS, Marra MA, Mayo M, Meng S, Meyerson ML, Mieczkowski PA, Moore RA, Mose LE, Mungall AJ, Pantazi A, Parfenov M, Park PJ, Parker JS, Perou CM, Protopopov A, Ren X, Roach J, Sabedot TS, Schein J, Schumacher SE, Seidman JG, Seth S, Shen H, Simons JV, Sipahimalani P, Soloway MG, Song X, Sun H, Tabak B, Tam A, Tan D, Tang J, Thiessen N, Triche T Jr, Van Den Berg DJ, Veluvolu U, Waring S, Weisenberger DJ, Wilkerson MD, Wong T, Wu J, Xi L, Xu AW, Yang L, Zack TI, Zhang J, Aksoy BA, Arachchi H, Benz C, Bernard B, Carlin D, Cho J, DiCara D, Frazer S, Fuller GN, Gao J, Gehlenborg N, Haussler D, Heiman DI, Iype L, Jacobsen A, Ju Z, Katzman S, Kim H, Knijnenburg T, Kreisberg RB, Lawrence MS, Lee W, Leinonen K, Lin P, Ling S, Liu W, Liu Y, Liu Y, Lu Y, Mills G, Ng S, Noble MS, Paull E, Rao A, Reynolds S, Saksena G, Sanborn Z, Sander C, Schultz N, Senbabaoglu Y, Shen R, Shmulevich I, Sinha R, Stuart J, Sumer SO, Sun Y, Tasman N, Taylor BS, Voet D, Weinhold N, Weinstein JN, Yang D, Yoshihara K, Zheng S, Zhang W, Zou L, Abel T, Sadeghi S, Cohen ML, Eschbacher J, Hattab EM, Raghunathan A, Schniederjan MJ, Aziz D, Barnett G, Barrett W, Bigner DD, Boice L, Brewer C, Calatozzolo C, Campos B, Carlotti CG Jr, Chan TA, Cuppini L, Curley E, Cuzzubbo S, Devine K, DiMeco F, Duell R, Elder JB, Fehrenbach A, Finocchiaro G, Friedman W, Fulop J, Gardner J, Hermes B, Herold-Mende C, Jungk C, Kendler A, Lehman NL, Lipp E, Liu O, Mandt R, McGraw M, Mclendon R, McPherson C, Neder L, Nguyen P, Noss A, Nunziata R, Ostrom QT, Palmer C, Perin A, Pollo B, Potapov A, Potapova O, Rathmell WK, Rotin D, Scarpace L, Schilero C, Senecal K, Shimmel K, Shurkhay V, Sifri S, Singh R, Sloan AE, Smolenski K, Staugaitis SM, Steele R, Thorne L, Tirapelli DP, Unterberg A, Vallurupalli M, Wang Y, Warnick R, Williams F, Wolinsky Y, Bell S, Rosenberg M, Stewart C, Huang F, Grimsby JL, Radenbaugh AJ and Zhang J. Comprehensive, integrative genomic analysis of diffuse lower-grade gliomas. N Engl J Med 2015; 372: 2481-2498.

[17] Mazur PK, Einwächter H, Lee M, Sipos B, Nakhai H, Rad R, Zimber-Strobl U, Strobl LJ, Radtke F, Klöppel G and Schmid RM. Notch2 is required for progression of pancreatic intraepithelial neoplasia and development of pancreatic ductal adenocarcinoma. Proc Natl Acad Sci U S A 2010; 107: 13438-13443.

[18] Fan X, Mikolaenko I, Elhassan I, Ni X, Wang Y, Ball D, Brat DJ, Perry A and Eberhart CG. Notch1 and notch2 have opposite effects on embryonal brain tumor growth. Cancer Res 2004; 64: 7787-7793.

[19] Maliekal TT, Bajaj J, Giri V, Subramanyam D and Krishna S. The role of Notch signaling in human cervical cancer: implications for solid tumors. Oncogene 2008; 27: 5110-4.

[20] Bajaj J, Maliekal TT, Vivien E, Pattabiraman C, Srivastava S, Krishnamurthy H, Giri V, Subramanyam D and Krishna S. Notch signaling in CD66+ cells drives the progression of human cervical cancers. Cancer Res 2011; 71: 4888-4897.

[21] Talora C, Sgroi DC, Crum CP and Dotto GP. Specific down-modulation of Notch1 signaling in cervical cancer cells is required for sustained HPV-E6/E7 expression and late steps of malignant transformation. Genes Dev 2002; 16: 2252-2263.

[22] Yeasmin S, Nakayama K, Rahman MT, Rahman M, Ishikawa M, Iida K, Otsuki Y, Kobayashi H, Nakayama S and Miyazaki K. Expression of nuclear Notch3 in cervical squamous cell carcinomas and its association with adverse clinical outcomes. Gynecol Oncol 2010; 117: 409-416.

[23] Virmani AK, Muller C, Rathi A, Zoechbauer-Mueller S, Mathis M and Gazdar AF. Aberrant methylation during cervical carcinogenesis. Clin Cancer Res 2001; 7: 584-589.

[24] K Kadian LK, Singhal G, Sharma S, Chauhan P, Nanda S and Yadav R. Incidence and association of HPV16 and 18 with various risk factors in cervical cancer patients in population of Haryana Region, India. J Clin Diagnostic Res 2019; 13: 10-13.

[25] Tripathi R, Rath G, Jawanjal P, Sharma S, Singhal P, Bhambhani S, Hussain S and Bharadwaj M. Clinical impact of de-regulated Notch-1 and Notch-3 in the development and progression of HPV-associated different histological subtypes of precancerous and cancerous lesions of human uterine cervix. PLoS One 2014; 9: e98642.

[26] Zur Hausen H. Papillomaviruses and cancer: from basic studies to clinical application. Nat Rev Cancer 2002; 2: 342-50.

[27] Baylin SB and Ohm JE. Epigenetic gene silencing in cancer-a mechanism for early oncogenic pathway addiction? Nat Rev Cancer 2006; 6: 107-16.

[28] Yang HJ. Aberrant DNA methylation in cervical carcinogenesis. Chin J Cancer 2013; 32: 42-8.

[29] Whiteside MA, Siegel EM and Unger ER. Human papillomavirus and molecular consider-

ations for cancer risk. Cancer 2008; 113: 2981-2994.
[30] Leonard SM, Wei W, Collins SI, Pereira M, Diyaf A, Constandinou-Williams C, Young LS, Roberts S and Woodman CB. Oncogenic human papillomavirus imposes an instructive pattern of DNA methylation changes which parallel the natural history of cervical HPV infection in young women. Carcinogenesis 2012; 33: 1286-1293.
[31] Franceschi S, Rajkumar T, Vaccarella S, Gajalakshmi V, Sharmila A, Snijders PJ, Munoz N, Meijer CJ and Herrero R. Human papillomavirus and risk factors for cervical cancer in Chennai, India: a case-control study. Int J Cancer 2003; 107: 127-133.
[32] Sowjanya AP, Jain M, Poli UR, Padma S, Das M, Shah KV, Rao BN, Devi RR, Gravitt PE and Ramakrishna G. Prevalence and distribution of high-risk human papilloma virus (HPV) types in invasive squamous cell carcinoma of the cervix and in normal women in Andhra Pradesh, India. BMC Infect Dis 2005; 5: 116.
[33] Datta P, Bhatla N, Pandey RM, Dar L, Patro AR, Vasisht S, Kriplani A and Singh N. Type-specific incidence and persistence of HPV infection among young women: a prospective study in North India. Asian Pac J Cancer Prev 2012; 13: 1019-1024.
[34] Senapati R, Nayak B, Kar SK and Dwibedi B. HPV genotypes distribution in Indian women with and without cervical carcinoma: implication for HPV vaccination program in Odisha, Eastern India. BMC Infect Dis 2017; 17: 30.
[35] Wang L, Qin H, Chen B, Xin X, Li J and Han H. Overexpressed active Notch1 induces cell growth arrest of HeLa cervical carcinoma cells. Int J Gynecol Cancer 2007; 17: 1283-1292.
[36] Talora C, Cialfi S, Segatto O, Morrone S, Choi JK, Frati L, Dotto GP, Gulino A and Screpanti I. Constitutively active Notch1 induces growth arrest of HPV-positive cervical cancer cells via separate signaling pathways. Exp Cell Res 2005; 305: 343-354.
[37] Zagouras P, Stifani S, Blaumueller CM, Carcangiu ML and Artavanis-Tsakonas S. Alterations in Notch signaling in neoplastic lesions of the human cervix. Proc Natl Acad Sci U S A 1995; 92: 6414-6418.
[38] Bolós V, Mira E, Martínez-Poveda B, Luxán G, Cañamero M, Martínez-AC, Mañes S and de la Pompa JL. Notch activation stimulates migration of breast cancer cells and promotes tumor growth. Breast Cancer Res 2013; 15: R54.
[39] Sun W, Gaykalova DA, Ochs MF, Mambo E, Arnaoutakis D, Liu Y, Loyo M, Agrawal N, Howard J, Li R and Ahn S. Activation of the NOTCH pathway in head and neck cancer. Cancer Res 2014; 74: 1091-1104.
[40] Steegenga WT, Boekschoten MV, Lute C, Hooiveld GJ, De Groot PJ, Morris TJ, Teschendorff AE, Butcher LM, Beck S and Müller M. Genome-wide age-related changes in DNA methylation and gene expression in human PBMCs. Age (Dordr) 2014; 36: 9648.
[41] Lu S, Niu Z, Chen Y, Tu Q, Zhang Y, Chen W, Tong W and Zhang Z. Repetitive element DNA methylation is associated with menopausal age. Aging Dis 2018; 9: 435-443.
[42] Choi JH, Park JT, Davidson B, Morin PJ, Shih IM and Wang TL. Jagged-1 and Notch3 juxtacrine loop regulates ovarian tumor growth and adhesion. Cancer Res 2008; 68: 5716-5723.
[43] Hu W, Lu C, Dong HH, Huang J, Shen DY, Stone RL, Nick AM, Shahzad MM, Mora E, Jennings NB and Lee SJ. Biological roles of the Delta family Notch ligand Dll4 in tumor and endothelial cells in ovarian cancer. Cancer Res 2011; 71: 6030-6039.