Clin Oncol Cancer Res (2009) 6: 401-405
DOI 10.1007/s11805-009-0401-7

Down-Modulation of Notch1 Expression in Cervical Cancer Is Associated with HPV-Induced Carcinogenesis

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Received September 30, 2009; accepted November 18, 2009.

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OBJECTIVE Notch1 signaling has been implicated in tumorigenesis. The purpose of this study was to investigate the putative role of the Notch1 receptor in carcinogenesis and in the progression of the cervical cancer. Since human papillomavirus (HPV) is a causative agent in cervical carcinoma, the interaction between Notch1 and HPV infection was examined.
METHODS Forty cervical cancer samples and 30 normal cervical tissue specimens were examined using Western blot and RT-PCR to detect Notch1 protein and mRNA levels. HPV16 DNA was examined in all samples using PCR.
RESULTS The level of Notch1 protein expression was significantly lower in cervical cancer tissue than in normal tissue. Levels of Notch1 mRNA were found to be substantially down-regulated in cancer tissue. Notch1 protein expression levels were significantly higher in carcinomas without HPV DNA than that in carcinomas with HPV infection (55.5% *vs.* 3.3%, $P < 0.05$). Down-modulation of Notch1 mRNA levels in carcinoma was demonstrated to be associated with HPVE6 transcription. Moreover, levels of Notch1 expression were shown to be significantly higher in early stage disease than in advanced stage disease ($P = 0.001$).
CONCLUSION Down-modulation of Notch1 expression probably plays an important role in the late stages of HPV-induced cervical cancer.

KEY WORDS: cervical cancer, Notch1, human papillomavirus (HPV).

Introduction

Cervical cancer is the second most common tumor among women in both incidence and mortality. HPV infection has been implicated as the most important etiologic factor in the development of cervical cancer. Although the link between HPV infection and cervical carcinogenesis is well confirmed, additional changes in host cells and in the surrounding environment are prerequisites for tumor development[1]. The incidence of cervical cancer is much lower than that of HPV infection. Therefore, HPV infection alone is considered insufficient for malignant conversion. The expression of viral oncogenes E6 and E7 in patients with high-risk HPV infection are always detected in the transformation of cervical epithelial cells. They can disrupt the p53 and Rb pathways, respectively. However, the disruption of the cell cycle and of apoptosis control through inactivation of p53 and Rb is also not considered to be sufficient for malignant transformation,

and the underlying molecular mechanisms are not fully understood. Several studies have investigated whether other proteins may be involved in the development of cervical cancer[2,3].

The evolutionarily conserved Notch gene family encodes transmembrane receptors of 300 kDa that are involved in the choice of cell fate. A number of reports suggest that Notch signaling may be involved in neoplastic transformation[4,5]. Struhl et al.[6], has proposed the proteolysis model of Notch signaling. The model indicates that ligand binding results in proteolytic cleavage of Notch and consequently releases the Notch intracellular domain (NICD). The molecule of the active form of Notch is 120 kDa in length and not full length Notch. The NICD then enters the nucleus and activates transcription of the target genes.

To investigate the putative role of Notch1 receptor in cervical cancer carcinogenesis, we analyzed the relationship between Notch1 and HPV. The goal of this study was to provide molecular evidence for developing individualized therapeutic plans for patients with cervical cancer.

Materials and Methods

Human cervical tissue samples

Forty samples of cervical squamous carcinoma and 30 samples of normal cervical tissue were obtained from patients who underwent surgery for cervical cancer in the Cancer Hospital/Institute of the Chinese Academy of Medical Sciences. After surgical removal, the specimens were frozen in liquid nitrogen for 1 h and then stored at -80°C until use. The clinical features of the specimens are described in Table 1.

DNA preparation and PCR

Genomic DNA in human cervical tissue was extracted using proteinase-K and phenol/chloroform as described previously[7]. DNA was dissolved in TE buffer and then stored at -20°C. Amplification for the HPV16 E6 region was performed with the primers (5'-CAA CAA GAC ATA CAT CGA CC-3' and 5'-CAA GCA ACA GTT ACT GCG A-3') based on the following conditions: at 95°C for 5 min; 35 amplification cycles at 94°C for 30 s, at 54°C for 30 s, and at 72°C for 45 s; and at 72°C for 2 min. The 321 bp PCR products were analyzed using 1.5% agarose gels and were photographed.

RNA isolation and semi-quantitative RT-PCR

Total RNA was isolated from cervical cancer tissue with Trizol reagent (Invitrogen) according to the manufacturer's instructions. A 1% agarose gel electrophoresis and spectrophotometry ($A_{260:280}$ nm ratio) were used to assess RNA quality. First strand cDNA was synthesized using random primer included in the Superscript II-reverse transcriptase kit (Invitrogen). Five microgramsμ of total RNA were used for each reaction, and 1 μl aliquots of the cDNA were then added to RT-PCR. The primers used for the amplification of Notch1 were as follows: sense 5'- GGC CAC CTG GGC CGG AGG TTA-3'; antisense 5'- GCG ATC TGG GAC TGC ATG CTG-3'. As an internal control, GAPDH was amplified to ensure the quality and quantity of cDNA for each RT-PCR.

Table 1. Clinical characteristics of 40 cervical squamous carcinomas.

Characteristics	No. of patients
Age (years)	
Mean	47.4
Range	28-66
Stage	
Ib1	6
Ib2	5
IIa	2
IIb	11
IIIb	15
IVb	1
Tumor size (cm)	
Mean	4.7
Range	3-9
Grade	
1	2
2	16
3	22
Pelvic lymph nodes (histopathologic diagnosis)	
Positive	5
Negative	11
Total	40

Western blot

Total cell lysates were abstracted from lysing cells in the buffer (1 % SDS, 10 mM Tris-Cl, pH 7.6, 20 μg/ml aprotinin, 2 μg/ml leupeptin and 1 mM PMSF). The protein concentrations were determined using the Bradford method (BIO-RAD, Hercules, CA). About 60 μg of protein were separated by 10% of SDS-PAGE and then transferred to PVDF membranes. After blocking with 10% non-fat milk, the membranes were incubated with anti-rabbit clonal antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA) at 4°C overnight. After performing 3-minute washes 6 times, the membranes were incubated with goat anti-mouse IgG at room temperature for 1 h. The signals were developed using the ECL kit (Amersham Pharmacia Biotechnology Inc, Milpitas, CA), and anti- mouse antibody (Santa Cruz) was employed as an internal control.

Statistical analysis

The correlations between the Notch1 expression and HPV were analyzed using Fisher's Exact Test. Chi-square tests were performed to determine the differences between normal epithelium and tumor tissue in Notch1 expression. Differences with a value of $P < 0.01$ were considered statistically significant.

Results

Expression of Notch1 in cervical cancer tissue

The results of examination by Western blotting on 40 samples of cervical cancer tissue and 30 samples of normal cervical epithelia showed that the expression of Notch1 protein was lower in cervical cancer tissue than that in normal cervical epithelia (Fig.1). Notch1 protein was expressed in a significantly lower percentage in cervical cancer tissue (6/40, 15%) than in normal tissue (100%) (Chi Square, $P < 0.01$). The results of statistical analysis correlating Notch1 protein expression in tumor tissue with clinicopathologic data showed that there were no significant correlations with age, sex and histologic differentiation.

The levels of Notch1 protein expression were significantly higher in early stage disease (I-IIa) than in the advanced stage (IIb-IV) (61.5% *vs.*7.4%) ($P = 0.001$, Table 2). This suggests that the loss of its expression was associated with the development of cervical cancer ($P = 0.001$).

Through the use of RT-PCR, we found that in 30 cervical cancer tissue samples the expression of Notch1 protein was absent and that there was lack of Notch1 mRNA in 29 of the samples (29/40). The 30 samples of normal cervical epithelia showed high levels of Notch1 expression (Fig.2). The expression of Notch1 protein and mRNA were similar, suggesting that the expression level of Notch1 mRNA was down regulated in cervical cancer. The levels of Notch1 mRNA were significantly higher in stages I-IIa (9/13, 69.2%) than in stages IIb-IV (2/27, 7%) ($P = 0.001$, Table 2). Through the progression from normal cervical epithelium to early stage to advanced stage cervical cancer, Notch1 expression became lower and lower. Thus, Notch1 might function as a tumor suppressor in the development of cervical cancer.

PCR analysis of HPVE6 infection

HPVE6 DNA was detected in 77.5% (31/40) cervical cancer tissue samples, but there was no amplification of HPVE6 DNA in the normal cervical epithelia (Fig.3).

Correlation between HPVE6 infection and Notch1 expression in cervical tissue

In 30 normal cervical tissue samples, there were no detectable HPV DNA, but Notch1 expression was positive. The results of this study indicated that the differential expression level of Notch1 protein was significantly higher in carcinomas without HPV DNA than that in carcinomas with HPV infection. The positive rate of the expression in these 2 groups was 55.5% and 3.3%, respectively ($P = 0.001$, Fig.4, Table 3). Taken together, these results suggest that a negative correlation between HPV infection and Notch1 expression may exist. Interestingly, down-modulation of Notch1 mRNA level in cervical carcinoma demonstrates that the level of Notch1 mRNA is associated with HPVE6 transcription.

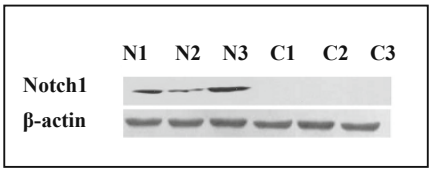

Fig.1. Western blot analysis of Notch1 protein expression, using β-actin as the internal control. The level of Notch1 protein expression was significantly lower in cervical cancer tissues than that in normal tissue samples. N, normal cervical tissue; C, cervical cancer tissue.

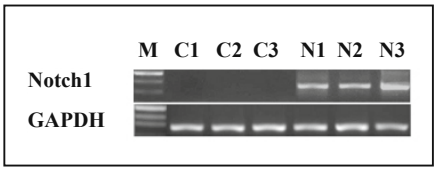

Fig.2. RT-PCR analysis of Notch1 expression, GAPDH was used as internal control. The levels of Notch1 mRNA were found to be substantially down-regulated in cervical cancer tissues. M, marker; N, normal cervical tissue; C, cervical cancer tissue.

Table 2. Notch1 protein and mRNA expression in cervical cancer tissues.

Stage	Protein expression (No. of patients)		*P*	mRNA expression (No. of patients)		*P*
	Negative	Positive		Negative	Positive	
I-IIa	4	9		5	8	
IIb-IV	25	2		25	2	
Total	29	11	0.001	30	10	0.001

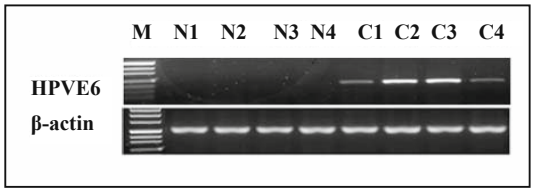

Fig.3. PCR analysis of HPVE6 infection in cervical tissues, using β-actin as the internal control. HPVE6 DNA was detected cervical cancer tissue samples but no amplification of HPVE6 DNA in normal cervical epithelia. M, marker; N, normal cervical tissue; C, cervical cancer tissue.

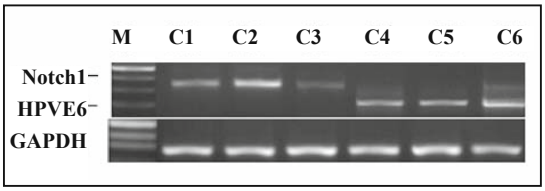

Fig.4. Correlation between HPVE6 infection and Notch1 expression in cervical tissues, GAPDH was used as internal control. The Notch1 protein differential expression level is significantly higher in the carcinomas without HPV DNA than in those with HPV infection. M: marker; N, normal cervical tissue; C, cervical cancer tissue.

Table 3. Correlation between HPVE6 infection and Notch1 expression in cervical tissue.

HPV	Notch1		Total
	Positive	Negative	
+	1 (3.3%)	30	31
-	5 (55.5%)	4	9
Total	6	34	40

Discussion

Cervical cancer represents a major type of keratinocyte-derived tumors. The findings from this study indicate that the expression of the Notch1 protein in cervical cancer tissue is not present.

Notch signaling is broadly involved in the development and in the function of many organs. Deletion of most of the extracellular domain as a consequence of a (9:7) chromosomal translocation results in the generation of a truncated Notch which is associated with a subgroup of human T-cell acute lymphoblastic leukemias. Many studies have demonstrated that loss of the extracellular subunit of Notch receptors can induce transformation. Aberrant expression of Notch receptors has been found in lung, renal and breast cancer[8-10]. However, there is mounting evidence that Notch signaling has more than just an oncogenic function, it can also act as a tumor suppressor. Among these, Notch1 not only plays an important role in the differentiation of normal cells but also is associated with the initiation and development of many tumors[11,12]. In this study, a 120 kDa Notch protein was detected through Western blot. The results of this study suggest that the expression of the Notch1 protein was lost in cervical cancer tissue, but not in normal cervical epithelia. Using RT-PCR, the loss of Notch1 expression can be seen at the mRNA level. A previous study showed that the increasing Notch1 expression that exists in precancerous lesions is caused by HPV infection[13]. The results from our study revealed a high expression level of Notch1 in normal cervical epithelium and a decreased expression level of Notch1 in invasive cervical cancer. Therefore, we think that in the progression from normal cervical epithelium to CIN to cervical cancer, Notch1 expression becomes lower and lower. That is to say that Notch1 might function as a tumor suppressor in the development of cervical cancer. The results of our study was similar to that reported by Talora and Yugawa[14-16].

One previous study demonstrated that high-risk HPV viral load was increased with the severity of cervical lesions. However, Notch1 expression was decreased in the progression from normal cervical epithelium to CIN to cervical cancer. The reduction of Notch1 activity led to a continuous expression of E6 and E7 genes in the HPV-infected cervical epithelium that induced its transformation. Some studies indicated that Notch1 exerts specific counteracting effects on HPV-induced transformation and on E6/E7 expression, and that down-modulation of Notch1 expression probably plays an important role in the later stages of HPV-induced carcinogenesis[15-17].

Cervical carcinoma is a major type of epithelial keratinocyte-derived tumor. Infection by human papillomavirus (HPV), more specifically the high-risk HPV16 and HPV18, is associated with most cervical cancers and is thought to be the cause of the disease[18]. HPVE6 DNA was detected in 77.5% (31/40) of the cervical cancer tissue samples, but no amplification of HPVE6 DNA was seen in the normal cervical epithelia. The results of our study were similar to that of a previous study and suggest that most HPV infections in cervical squamous carcinoma patients were HPV16. In the normal cervical epithelia used as controls, there was no detectable HPV16.

Although it has been found that there is a close relationship between Notch1 expression and HPV, not all the HPV16-negative cervical cancer has normal expression levels of Notch1. There was no Notch1 expression in 4 of the HPV16-negative cervical cancer cases. This might be caused by other types of HPV infection in these samples that led to the loss of Notch1 expression. Notch1 expression can be detected in HPV16-positive cervical cancer tissue, suggesting that down-modulation of Notch1 expression is likely to play an important role but not the only role in the later stages of HPV-induced carcinogenesis[19].

In conclusion, Notch1 may exert protective effects

on HPV-induced transformation. Down-modulation of Notch1 expression is likely to play an important role in the late stages of HPV-induced carcinogenesis[20]. Whether Notch1 expression can help in the preoperative diagnosis and in the planning of individual therapies for cervical cancer patients requires further investigation.

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