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Occurrence and Expression of Human Papillomavirus Type 16 Genes in Uterine Cervical Carcinomas

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ABSTRACT

The detection of human papillomavirus (HPV) type 16 early genes: E7, E5, and the late gene: L1 was attempted in 42 uterine cervical neoplasia (35 cervical carcinomas and 7 cervical dysplasias) using the polymerase chain reaction (PCR) method. Consequently, E7 gene was detected in 19 (54.3%) of 35 carcinomas and in 5 (71.4%) of 7 dysplasias, E5 gene was detected in 7 (20.0%) of 35 carcinomas and in 5 (71.4%) of 7 dysplasias, L1 gene was detected in 18 (51.4%) of 35 carcinomas and in 5 (71.4%) of 7 dysplasias, respectively.

In order to elucidate the transcriptional pattern of HPV type 16 in each of the clinical stages, the expression of mRNA for E7, E5 and L1 genes was examined in HPV DNA positive cases using the reverse transcriptase polymerase chain reaction (RT-PCR) method. E7 gene mRNA was detected in 18 (94.7%) of 19 cervical carcinomas, whereas E5 and L1 genes mRNAs were detected in only 4 (57.1%) of 7 and in one (5.6%) of 18 carcinomas respectively. In cervical dysplasias, E7, E5 and L1 genes mRNA were detected in all cases. E7, E5 and L1 genes were transcriptionally active in all dysplasias, whereas E5 and L1 genes were not always transcriptionally active in carcinomas.

These results suggest that the HPV type 16 early gene E7 is present preferentially as integrated form and transcriptionally active in the carcinoma cell, and plays an important role in the development of malignancy. On the other hand, E5 and L1 genes are present and transcribed in the dysplasia cell but their transcriptional activity is less frequent in the carcinoma cell.

Key words: *HPV, Uterine cervical carcinoma, Reverse transcriptase polymerase chain reaction, Gene expression*

Human papillomavirus (HPV) is a member of the papovavirus family and known as an etiologic agent of skin and mucocutaneous tumorigenesis in the human anogenital area. Recent investigations have revealed that some types of HPV, mostly type 16 and 18, are demonstrated frequently in uterine cervical neoplasia and associated with carcinogenesis of uterine cervix[4,7,22,24]. There has been increasing evidence that HPV DNA exists as an integrated form in advanced carcinoma, whereas it commonly exists as an episomal form in dysplasia[5,23]. However, little is known about the function and significance of each of the integrated HPV genes in carcinogenesis of the uterine cervix.

In view of these findings, it is important to analyze the physical state of HPV genomes and the alteration of their transcriptional pattern at each stage of malignancy. This study was undertaken to fulfill two purposes. The first aim was to examine the existence of HPV type 16 early genes: E7, E5; and late gene: L1 DNA in uterine cervical neoplasia by polymerase chain reaction (PCR), and the second was to analyze the expression of mRNA of these genes by reverse transcriptase polymerase chain reaction (RT-PCR) to ascertain whether or not integrated viral sequences are actively transcribed. In addition, Northern blot hybridization which is the standard method for analysis of mRNA expression was compared with RT-PCR to determine their usefulness in the detection of HPV type 16 mRNA.

MATERIALS AND METHODS

Tissue specimens: Cervical carcinoma and dysplasia specimens were taken from forty-two patients who underwent abdominal total hysterectomy in the Department of Obstetrics and Gynecology, Hiroshima University School of Medicine (Table 1). These included 8 stage II and 14 stage I cervical carcinomas, 13 carcinomas in situ (CIS) and 7 severe dysplasias. Tissue specimens were frozen in liquid nitrogen immediately after surgical removal and stored at -80°C until use. A part of the tissue was fixed in 10% formalin and diagnosed histopathologically.

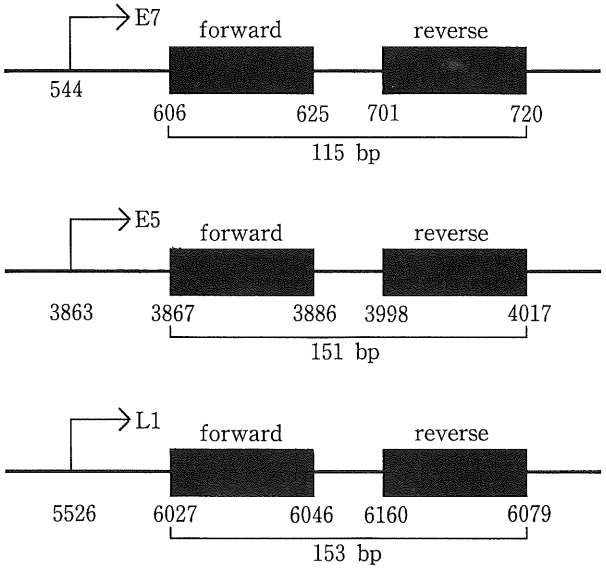
Table 1. Clinical features and results of detection of HPV type 16 DNA (mRNA) in subjects studied

case	age	stage	histology	HPV 16 DNA (mRNA*)		
				E7	E5	L1
1	31	dys	–	+(+)	+(+)	+(+)
2	43	dys	–	+(+)	+(+)	+(+)
3	42	dys	–	+(+)	+(+)	+(+)
4	35	dys	–	–	–	–
5	41	dys	–	–	–	–
6	54	dys	–	+(+)	+(+)	+(+)
7	33	dys	–	+(+)	+(+)	+(+)
8	30	0	Lc	+(+)	–	+(–)
9	35	0	Lc	+(+)	–	+(–)
10	46	0	Lc	+(+)	+(–)	+(–)
11	43	0	Lc	+(+)	–	+(–)
12	37	0	Lc	+(+)	–	+(–)
13	51	0	Lc	+(+)	+(–)	+(–)
14	49	0	Lc	–	–	–
15	49	0	Lc	–	–	–
16	40	0	Lc	+(+)	–	+(–)
17	42	0	Lc	+(+)	+(+)	+(+)
18	61	0	Lc	–	–	–
19	47	0	Lc	+(+)	–	+(–)
20	73	0	Lc	–	–	–
21	33	Ia	Ade	–	–	–
22	42	Ia	LNK	+(+)	+(+)	+(–)
23	41	Ia	LNK	–	–	–
24	62	Ia	LNK	–	–	–
25	55	Ib	LNK	+(+)	–	+(–)
26	38	Ib	LK	+(+)	–	–
27	42	Ib	LNK	+(+)	–	+(–)
28	66	Ib	LNK	+(+)	–	+(–)
29	41	Ib	LK	+(+)	+(+)	+(–)
30	62	Ib	Ade	–	–	–
31	43	Ib	LNK	–	–	–
32	47	Ib	LNK	+(+)	+(+)	+(–)
33	54	Ib	LNK	+(–)	+(–)	+(–)
34	53	Ib	LNK	–	–	–
35	64	IIa	LK	–	–	–
36	42	IIa	Ade	–	–	–
37	58	IIb	LK	–	–	–
38	54	IIb	LNK	+(+)	–	+(–)
39	47	IIb	LNK	–	–	–
40	69	IIb	LNK	–	–	–
41	39	IIb	LNK	+(+)	–	+(–)
42	60	IIb	LK	–	–	–

LNK: squamous cell carcinoma large cell non-keratinizing type
LK: squamous cell carcinoma large cell keratinizing type
Ade: adenocarcinoma, Lc: carcinoma in situ large cell type
* Detection of HPV type 16 mRNA by RT-PCR shown in parentheses

DNA preparation: DNA was extracted from formalin-fixed paraffin-embedded blocks of surgical specimens[2]. The blocks were cut into sections 10 μm thick and 10 or more sections were collected. One section was saved for microscopic examinations. After deparaffinization by 100% xylene and rehydration by 100% and 90% ethanol, the specimen was suspended in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 100 mM NaCl containing 0.5% sodium dodecyl sulfate (SDS) and proteinase K (200 μg/ml) at 37°C for 2 days. Subsequently, DNA was extracted with phenol and then with a mixture of 24 parts of chloroform and one part of isoamyl alcohol. Sodium acetate was added to each sample and DNA was precipitated by 2.5 volumes of ethanol. The specimen was kept overnight at –30°C, and after centrifugation the pellet was washed with 80% ethanol and resuspended in H_2O.

PCR: The PCR was performed with Gene-Amp Kit (Perkin-Elmer Cetus, Norwalk, CT. U.S.A.).



E7 forward : 5'GCAACCAGAGACAACTGATC
E7 reverse : 5'ATTGTAATGGGCTCTGTCCG

E5 forward : 5'GCATCCACAACATTACTGGC
E5 reverse : 5'AACGCAGAGGCTGCTGTTAT

L1 forward : 5'AATGCTAGTGCTTATGCAGC
L1 reverse : 5'ATTTACTGCAACATTGGTAC

Fig. 1. Sequence of E7, E5 and L1 primers and locations in HPV type 16 genome.

The E7, E5 and L1 primers of HPV type 16 are shown in Fig. 1. These primers were synthesized by a DNA synthesizer (Wakunaga Pharm. Co.). L1 primers were made according to the sequence described by Cornelissen et al[12]. PCR using the E7, E5 and L1 primers yielded 115bp, 151bp and 153bp fragments, respectively.

One microgram of extracted DNA was denatured at 94°C for 10 min, and added to a 50 μl of PCR solution (10 mM Tris-HCl pH8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin) containing 0.2 mM each of dATP, dGTP, dCTP, dTTP, 0.2 μM each of the primers (forward, reverse) and 2.5 units of Taq-DNA polymerase (TAKARA). The mixture was incubated at 94°C for 1 min to denature the DNA, the primers were allowed to anneal to the DNA at 55°C for 2 min and extension was performed at 72°C for 2 min. Thirty cycles of amplification were repeated by Thermal Cycler (Perkin-Elmer Cetus). After amplification, 10 μl of the reaction mixture was subjected to electrophoretic analysis on 3% NuSieve agarose (FMC, Rockland, ME. U.S.A.) and stained with ethidium bromide.

RNA preparation: RNA was extracted by the guanidium isothiocyanate / cesium chloride method[11]. Solution 1 (4M guanidinium isothiocyanate, 5mM sodium citrate pH 7.0, 0.5% sarkosyl and 1% 2-mercaptoethanol) was added to tissue specimens and dispersion was performed by homogenizer. One gram of cesium chloride was added to each 2.5 ml of homogenate and the homogenate was layered onto 2.5 ml of 5.7M CsCl in 0.1M EDTA (pH 7.5) in a centrifuge tube. Centrifugation was performed at 35,000 rpm for 14 hours at 20°C in a Beckman SW-40. After the supernatant was discarded, the pellet of RNA was dissolved in solution 2 (10 mM Tris-HCl, pH 7.4, 5mM EDTA and 1% SDS). A mixture of chloroform and 1-butanol (4:1) was added and the aqueous phase was transferred to a fresh tube. Three molar sodium acetate and 2.2 volumes of ethanol were added to the tube which was stored overnight at -20°C. The RNA was recovered by centrifugation and dissolved in H_2O.

RT-PCR: The reverse transcription of RNA was performed with AMV Reverse Transcriptase System (BRL, Gaithersburg, U.S.A.) and the PCR was performed with Gene-Amp Kit. Two point five microliters of nucleotide solution (10mM dATP, dGTP, dTTP and dCTP in 10mM Tris-HCl, pH 7.5) was added to 2μg of RNA and lyophilized, and then the following solutions were added to the samples: 5μl of 250mM KCl, 5μl of reverse transcription buffer (500mM Tris-HCl, pH 8.3, 50mM $MgCl_2$, 50mM DTT), 5μl of 100 μg/ml oligo $dT_{12\text{-}18}$ solution, 5μl of 250 μg/ml actinomycin D solution, 5 units of AMV reverse transcriptase. The reaction mixture was incubated at 37°C for 1 hour, and 2μl of the sample was subjected to PCR.

Northern blot hybridization: Ten micrograms of total RNA from 30 cases were electrophoresed on 1% agarose gel containing 6% formaldehyde and blotted onto nitrocellulose filters. Filters were baked for 2 hours at 80°C under vacuum. After pre-hybridization, hybridization was performed at 42°C for 16 hours. The hybridization solution contained 0.1M PIPES-NaOH, pH6.8, 0.65M NaCl, 5×Denhardt's solution (1×Denhardt's consisted of 0.02% (w/v) each of bovine serum albumin, Ficoll, and polyvinyl pyrrolidine), 0.1% SDS, 50% deionized formamide, 10% Dextran sulfate and 100 μg/ml salmon sperm DNA.

The HPV type 16 DNA probe was labeled with [^{32}P]-dCTP by multiprime DNA labeling system (Amersham, Tokyo, Japan).

After hybridization, filters were washed twice in 0.1×SSC-0.1%SDS (1×SSC is 0.15M NaCl and 0.015M sodium citrate) for 30 min at room temperature, then washed twice in 0.1×SSC-0.1%SDS for 60 min at 65°C and rinsed in 0.1×SSC. Filters were autoradiographed overnight at -80°C with Kodak XAR-5 films with an intensifying screen.

Slot blot hybridization: Five microliters of the PCR and RT-PCR product were transferred to a

nitrocellulose filter membrane. Hybridization and washing procedures were performed as described above.

RESULTS

Analysis of the presence of E7, E5 and L1 DNAs of HPV type 16 were performed in 42 uterine cervical neoplasia cases (Table 1) and representative cases among these are shown in Fig. 2. The 115bp and 153bp bands of E7 and L1 DNA in case 16, and 115bp, 151bp and 153bp bands in case 17 were demonstrated. The percent positive for E7, E5 and L1 in each stage of cervical neoplasia are summarized in Table 2. In the cases of dysplasias that were positive in viral DNA, all of the E7, E5 and L1 genes were detected simultaneously, whereas E5 was not always detected in the viral DNA-positive carcinoma cases. The frequency of HPV type 16 DNA in the advanced cervical carcinomas (stage II) was lower (E7: 25.0%, E5: 0%, L1: 25.0%) than those in the early stages (stage 0: E7: 69.2%, E5: 23.1%, L1: 69.2% and stage I: E7:

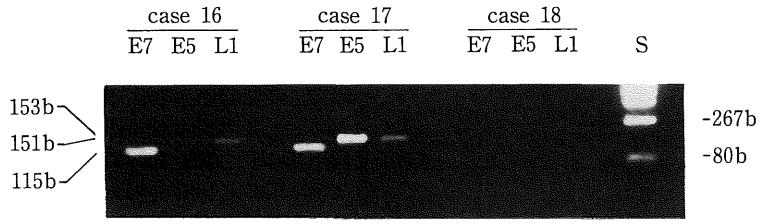


Fig. 2. Detection of E7, E5 and L1 DNAs of HPV type 16 in uterine cervical carcinoma. After PCR, 10μl of the sample was subjected to electrophoretic analysis and stained with ethidium bromide as described in Materials and Methods.

Table 2. Frequency of E7, E5 and L1 DNA detected in cervical neoplasia by clinical stage

	E7	E5	L1
dysplasia	71.4% (5/7)	71.4% (5/7)	71.4% (5/7)
carcinoma	54.3% (19/35)	20.0% (7/35)	51.4% (18/35)
stage 0	69.2% (9/13)	23.1% (3/13)	69.2% (9/13)
stage I	57.1% (8/14)	28.6% (4/14)	50.0% (7/14)
stage II	25.0% (2/8)	0% (0/8)	25.0% (2/8)

Stages in carcinoma according to the criteria of the International Federation of Gynecology and Obstetrics (1971). Number of positive/total shown in parentheses.

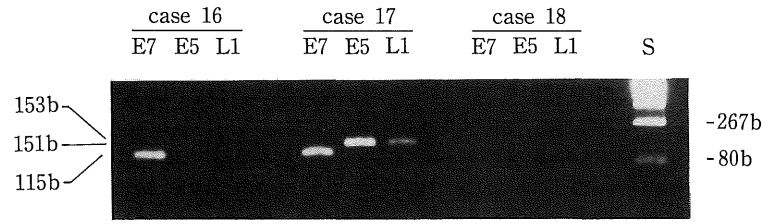


Fig. 3. Detection of E7, E5 and L1 mRNAs of HPV type 16 by RT-PCR. After amplification by RT-PCR, 10 μl of the sample was subjected to electrophoretic analysis and stained with ethidium bromide as described in Materials and Methods.

Table 3. Expression of mRNA for each gene in HPV-DNA positive cases

	E7	E5	L1
dysplasia	100% (5/5)	100% (5/5)	100% (5/5)
carcinoma	94.7% (18/19)	57.1% (4/7)	5.6% (1/18)
stage 0	100% (9/9)	33.3% (1/3)	11.1% (1/9)
stage I	87.5% (7/8)	75.0% (3/4)	0% (0/7)
stage II	100% (2/2)	– (0/0)	0% (0/2)

See footnotes to Table 2.

57.1%, E5: 28.6%, L1: 50.0%).

To elucidate the expression of the viral genes in carcinomas of each clinical stage, mRNAs for E7, E5 and L1 genes were examined by RT-PCR for the cases of viral DNA positive (Fig. 3 and Table 3). E7 mRNA was detected in 18 (94.7%) of 19 E7 DNA positive carcinomas, while E5 and L1 mRNAs were detected only in 4 (57.1%) of 7 and in one (5.6%) of 18 carcinomas positive for these DNAs, respectively. In cases of dysplasia, E7, E5 and L1 mRNA were detected in all of the HPV DNA positive cases. HPV mRNA was not detected in the HPV DNA negative cases.

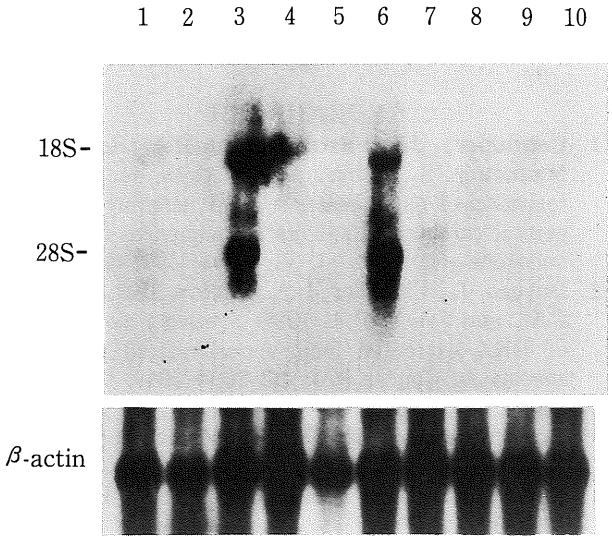


Fig. 4. HPV type 16 mRNA detected by Northern blot hybridization. The method is described in Materials and Methods. Lanes: 1—10; case 11, 12, 22, 23, 13, 29, 14, 15, 16 and 30 respectively. β-actin probe applied as an internal control.

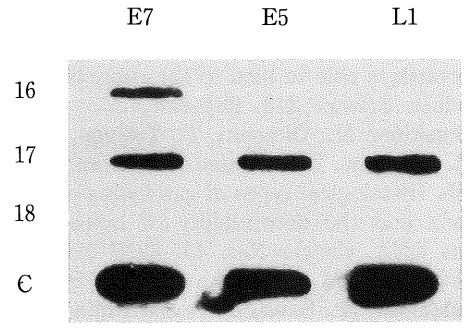


Fig. 5. Slot blot hybridization of RT-PCR product. A 5μl volume of the sample was applied to nitrocellulose filter, hybridized with ^{32}P labeled probe DNA, washed, and autoradiographed as described in Materials and Methods. The three upper lanes (16, 17, 18) correspond to case 16, 17 and 18 in Table 1. E7 in case 16 and E7, E5 and L1 in case 17 showed positive signals, which corresponded to the results of RT-PCR. The PCR products from HPV type 16 DNA were used as a positive control (lane C).

In Northern blot hybridization analysis, HPV type 16 mRNA was detected in 6 cases (8, 9, 10, 17, 22, 29) of 30 cervical carcinomas (from case 8 to case 30 and from case 35 to case 41 in Table 1). Representative cases are shown in Fig. 4. Two major transcripts of about 4.2kb and 1.8kb and some minor transcripts were detected in the HPV type 16 positive cases. These bands corresponded to the previously reported HPV type 16 transcripts[14]. The β-actin probe was employed as an internal control. Transcription of viral genes was detectable in only 6 (20.0%) cases among these 30 cervical carcinomas by Northern blot hybridization, while 17 (56.7%) cases were positive by RT-PCR.

Slot blot hybridization was applied to confirm whether the PCR and the RT-PCR products were derived from the HPV type 16 genes (Fig. 5). The results of slot blot hybridization revealed that all the cases positive in PCR and RT-PCR were also positive in the slot blot hybridization.

DISCUSSION

By analyzing HPV type 16 DNA in uterine cervical neoplasia, it was demonstrated that all of the E7, E5 and L1 genes were detected in 71.4% of dysplasia, while the frequencies of detection of E7, E5 and L1 genes decreased and varied in carcinomas. E7 (54.3%) and L1 (51.4%) genes were relatively well conserved in the carcinoma cells than E5 gene (20.0%). These findings may support the hypothesis that HPV type 16 DNA is integrated into the cellular DNA in carcinoma, while in dysplasia, HPV DNA is present as a complete viral gene in the episomal state[8,15,16]. In view of the association of HPV type 16 DNA with clinical stage of uterine cervical neoplasia, the advanced carcinoma showed a lower frequency of HPV type 16 DNA (Table 2). This result suggests that HPV type 16 may be not related to maintenance of malignancy, but related to initiation of the process.

Futhermore, in the study to elucidate the transcriptional pattern associated with clinical stages, the expression of mRNA for E7, E5 and L1 genes was transcriptionally active in all the dysplasia cases. As for the carcinoma cases, E7 gene was transcribed in all E7 DNA positive cases with the exception of one case of stage I carcinomas. The reason why E7 gene was not transcribed in this case is unclear.

In this study, RT-PCR was used to analyze HPV type 16 mRNA, since it was difficult to obtain sufficient amounts of RNA from uterine cervical neoplasia. In order to evaluate the utility of RT-PCR for detection of HPV type 16 mRNA, the sensitivity of RT-PCR was compared with that of Northern blot hybridization. HPV type 16 mRNA was detected in 17 (56.7%) of 30 cases by RT-PCR, whereas in only 6 (20.0%) of 30 cases by Northern blot hybridization. These results show that RT-PCR is

more sensitive than Northern blot hybridization and that RT-PCR is very useful in detection of HPV mRNA from small specimens.

E7 gene is considered to be the most well retained among HPV type 16 genes in cervical carcinoma[20], and in the course of development of malignancy E7 gene is transcribed actively even though the alteration of transcriptional regulation might occur[14,17]. Wilczynski et al[20] have suggested that E7 gene plays a role in the maintenance of the malignant state, while, Lehn et al[10] have reported that HPV type 16 may be a potential hazard in the first step of the multistep development of cervical carcinomas and that the maintenance of the malignant nature may not depend on the continuous transcriptional activity of HPV type 16. In the present study, E7 DNA was less frequently detected in advanced carcinoma, while transcriptional activity of this gene was well conserved in all stages of carcinoma. These results suggest that E7 gene might be an initiator in the early stages of malignancy. The other early and late genes, such as E5 and L1, were less retained than E7 and transcriptionally unstable in carcinoma. E5 and L1 genes may be present as episomal form and transcriptionally active in dysplasia. After integration they might become transcriptionally inactive and subsequently deleted in carcinomas. These findings suggest that E5 and L1 are not essential to the maintenance of the malignant state. The other genes should also be examined to clarify which genes of HPV type 16 are important in malignant conversion and maintenance of malignancy.

Recent investigations have revealed that multistep genomic alteration may induce the malignant conversion of cells, and HPV type 16 plays an important role in the carcinogenesis of uterine cervix not only by its own transforming activity[9,13,18], but also by association with alteration of the oncogene and/or the tumor suppressor gene[21]. In some cervical carcinomas, HPV genomes have been integrated in regions of the human genome near the c-*myc* gene; cis-activation of the c-*myc* gene by inserted viral sequences might be involved in malignant transformation[1,3]. Retinoblastoma (RB) gene product has been shown to form complexes with the HPV type 16 E7 protein. Consequently, inactivation of the RB protein which has tumor suppressor properties might occur[6], and the HPV type 16 E6 protein has been shown to bind to p53 protein, which is also tumor suppressor gene product, in a similar manner[19]. Therefore the investigation of the integration and expression of E6/E7 genes must be the most important elements in clarifying the role of HPV type 16 in multistep carcinogenesis and development of malignancy in the uterine cervix.

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