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Loss of heterozygosity of chromosome 3p in patients with carcinoma of the cervix

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Loss of heterozygosity (LOH) at chromosome 3p frequently occurs in squamous cell carcinomas of the uterine cervix and indicates the probable sites of tumor suppressor genes that play a role in the development of this tumor. To study such recessive mutations involved in multistep cervical carcinoma, we separated tumors and normal DNA pairs from 43 invasive squamous cell carcinomas of the cervix on archived slides. We studied LOH using polymerase chain reaction (PCR) with 10 polymorphic DNA marker on the 3p12-26.1 region. HPV type 16 and 18 were preformed by PCR on tumor specimens. LOH at one or more loci was observed in 15 cases (35%). HPV DNA of any types was detected in 35 cases of all tumors (81%). HPV 16 was detected in 32 cases (74%) and HPV 18 in 9 cases (21%). In the 32 cases with positive HPV 16 infection, 9 (28%) had LOH, while in the 11 with negative HPV 16 infection, 6 (55 %) had LOH. Three cases in the 9 positive HPV 18 infection (33%) had LOH, while 12 in the 34 negative HPV 18 infection (35%) had LOH. Our results confirm that no relationship was found between 3p LOH and HPV.

Key words; *cervical carcinoma, chromosome 3p, loss of heterozygosity, microsatellite, human papillomavirus*

Introduction

Invasive cervical cancer is the second most common malignancy in women worldwide, accounting for about 500,000 cases per year.[1] Cancer of the uterine cervix is associated with human papillomavirus (HPV). HPV infection appears to play an important role in cervical carcinogenesis, though HPV infection alone might not be capable of developing cervical cancer.[2]

In addition to the above host-viral interactions, other factors must be important in cervical tumorigenesis. Loss of heterozygosity (LOH) of specific chromosomes or chromosome segments occurs in many neoplasias and generally indicates the sites of tumorigenicity suppressor-like genes.[3] Therefore, the chromosomal location of candidate tumor suppressor genes can be inferred through detection of LOH at specific loci. LOH or allelic imbalance is observed as a complete or partial signal reduction of the two corresponding alleles in the matching tumor DNA. Such chromosome losses can be detected by polymorphic markers.

The most frequent area of chromosomal deletion was the 3p12-14.1 region. Other investigators have shown frequent allelic loss in several chromosome 3p regions. Kohno et al. (1993) showed 45% loss in chromosome 3p of 47 cervical cancers.[4] Chung et al. (1992) report an 83% LOH at 3p25 and 90% LOH at 3p13 in 12 cervical carcinomas.[5] Jones et al.

책임저자 : 김진우

(1994) also report a very high frequency of LOH at 3p13-21 in a small sample of eight cervical tumors.[6] Mitra et al. (1994) had 40% loss at 3p21.33-22.1 in 30 informative cervical tumors.[7]

In this study, we analyzed the presence of LOH on chromosome 3 for 10 polymorphic markers and HPV infection in 43 women with squamous cervical carcinomas. We also examined the relationship between the results of the status of LOH and HPV infection status.

Materials and Methods

Histologic materials

Forty-three women with carcinoma of the cervix were selected from 1996 to 2000. All the cancer cases were confirmed squamous cell carcinoma (stage Ib1, Ib2, stage IIa). The proportion of tumor cells varied from 60% to 90%. The normal cervical epithelium used in this study was adjacent to tumor tissue and was verified as normal by histopathologic analysis.

Microdissection and DNA extraction

Two 10-㎛ sections were cut from each tissue block. Slides were deparaffinized in zylene for 5 minutes and taken through increasingly hydrated alcohol. Each section was then stained with methylene blue for 10 seconds, washed with running distilled water for a few minutes and dried. Areas of lesions and normal tissue were dissected directly from the stained slides under the microscope by fine surgical needles. Precautions were taken to prevent cross-contamination.

Microdissected lesions and normal tissues were transferred to 1.5 ㎖ Eppendorf tubes with 200 ㎕ of 100% ethanol. The samples were centrifuged at 2000 g for 10 minutes. After discarding the supernatant, 200 - 400 ㎕ digestion buffer (50 mM Tris (pH 8.0), 1 mM EDTA, 0.5% Tween 20 and 500 ㎍/㎖ of proteinase-K) was added and incubated overnight at 56 ℃. After completion of proteinase-K digestion, the proteinase-K was heat-inactivated at 95 ℃ for 10 minutes.[8]

LOH detection with PCR technique

Primer sequences flanking 10 polymorphic microsatellite loci mapped to chromosome 3p were obtained from the Genome Date Base (http://www.gdb.org/gdb/map). We restricted our analysis to a small number of markers because some of our samples contained a very limited amount of DNA. Loci were grouped on the basis of primer sequence, amplified allele size and chromosomal location at Table 1.

One 2-㎕ aliquot of each paired DNA sample was amplified in 20 ㎕ of polymerase chain reaction (PCR) mixture containing 200 ㎛M each dNTP, 0.4 unit of Taq polymerase (BM, USA), 10X buffer, 1.5 mM MgCl2, and 20 pM of each primers. After PCR amplification, gel electrophoresis was carried out using 12% polyacrylamide gel (29:1 acrylamide:bisacrylamide). Gels were stained with ethidium bromide and photographed under ultraviolet light. Heterozygosity was defined by two clearly prominent bands of the expected size range in the normal tissue. LOH was scored by visual inspection. Due to contamination of tumor samples by constitutive cells, LOH often presented as a reduction in the intensity of the corresponding band compared to the constitutive DNA.

Detection of HPV

The HPV typing of the cases was done without knowledge of the clinical followup information. The HPV sequenced in paraffin-extracted tumor DNA were amplified by PCR using type 16 and 18 primers (Table 1). In each PCR run, positive and negative controls were used.

Table 1. Microsatellite markers and PCR

Locus	Primer sequence (5'→3')	Map position	Size range of PCR product
D3S1038	TCCAgTAAgAggCTTCCTAg AAAggggTTCAggAAACCTg	3p26.1-3p25.2	115-141
D3S1110	TCGACATCAGAATGCCCTATAC GGTGTCTGCCACTGTTCTGGG	3p25.3-3p25.1	68-74
D3S1029	ATACTCTggACCCAgATTgATTAC TAATTCCCAAATggTTTAggggAg	3p21.31-3p21.2	160-174
D3S1076	ATTCCCTgCATATgATCCCAACTg ACCTAAgAgCAATCACTTAACTTAg	3p21.2-3p21.1	119-133
D3S1295	TgTAgTAATggTTTCATggATACAC ATTTTATAAgTTTTgATACCCACCC	3p21.1-3p14.2	120-150
D3S1592	AggCAgAggCTACAgTATTg TgTgTTgATTTCgTATCCTg	3p21.1-3p14.2	270-290
D3S1313	CCCCTTGGAAAATCACTG CCATgAATAAgCCTTgCC	3p21.1-3p14.2	220-240
D3S1228	TCTTAACTCTTTCTCTgTgAgTTg TCTAggAAAgggATTAggAAggA	3p14.2-3p14.1	78-96
D3S1287	ATAACACAACAAgCAAgCCTATggT gAgTgACATTTgCCCCTTTg	3p14.2-3p14.1	255-263
D3S1284	GCCTTGGGGGTAAATACTCT GGAATTACAGGCCACTGCTC	3p12-3p12	155-178
HPV 16	AAgggCgTAACCgAAATCggT gTTTgCAgCTCTgTgCATA		140
HPV 18	AAgggCgTAACCgAAATCggT gTgTTCAgTTCCgTgCACA		140

Results

LOH analysis was performed for 43 patients with invasive squamous cell carcinomas of the cervix using 10 polymorphic markers distributed over chromosome 3p (Table 1). PCR results from successfully amplified cases are shown in table 2.

Fifteen cases (35%) had LOH in at least one of the ten chromosomal regions. Figure 1 shows representative gel demonstrating LOH at several loci. The most frequent chromosome 3p regions affected by LOH was 3p25.1-3p25.3 (D3S1110, 12%) and 3p21.1-3p14.2 (D3S1592, 11%). No LOH was found at locus D3S1295 marker in any case (Table 2).

All samples harbored the oncogenic HPV sequences: HPV DNA of any types was detected in 35 cases of all tumors (81%). HPV 16 and 18 sequences were detected in 74% (32/43) and 21% (9/43), respectively. Among them, 6 cases (14%) were coinfected with both HPV types (Table 2 and Fig. 2).

In the 32 cases with positive HPV 16 infection, 9 (28%) had LOH and 23 were not found LOH. While in the 11 with negative HPV 16 infection, 6 (54%) had LOH. Three cases in the 9 positive HPV 18

infection (33%) had LOH and 6 were not found LOH. While 12 in the 34 negative HPV 18 infection (35%) had LOH. Associations between the LOH of chromosome 3p and HPV infection were not significantly associated because LOH rates of all cases carried HPV 16 and 18 sequences was 28% and 33%, respectively.

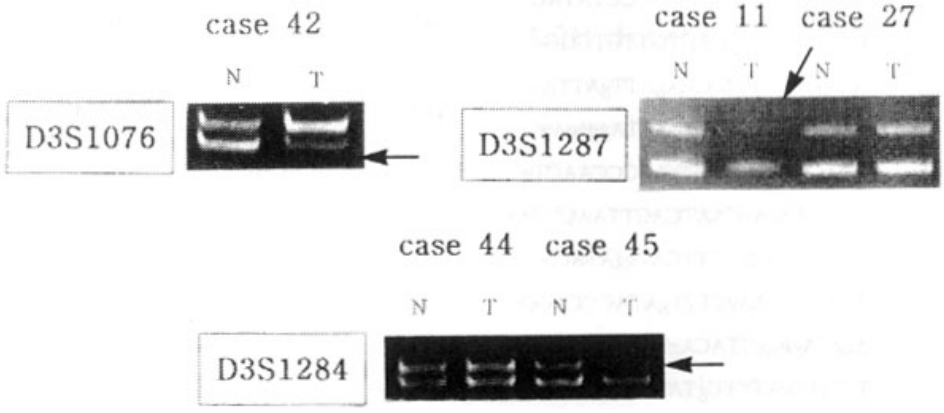

Fig. 1. Representative results of LOH analysis at chromosome 3p in cervical carcinoma. Arrowheads indicate the absent or decreased allele in tumor. N, normal tissue; T, tumor tissue.

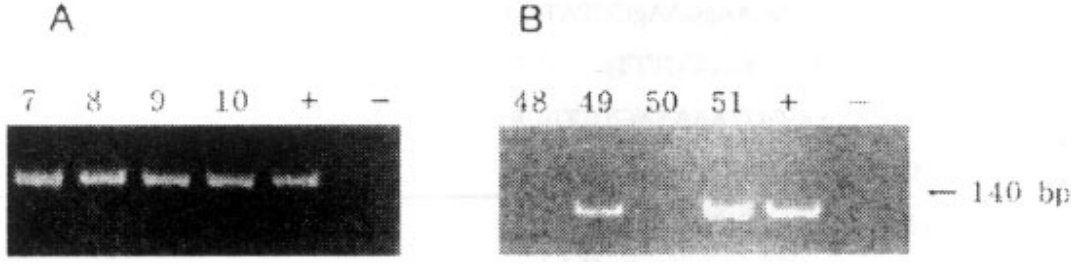

Fig. 2. Representative gel of HPV 16 (A) and 18 (B) in cervical carcinomas. (A) HPV 16 in case 7, 8, 9 and 10; (B) HPV 18 in case 48, 49, 50, and 51. +, positive controls; -, negative controls.

Table 2. LOH at 3p12-26.1 and HPV infection in cervical carcinomas (43 cases)

case number	D3 S1038	D3 S1110	D3 S1029	D3 S1076	D3 S1295	D3 S1592	D3 S1313	D3 S1228	D3 S1287	D3 S1284	HPV 16	HPV 18
1	○	○	○	○	○	○	○	●	○	○	-	-
3	○	○	○	○	○	○	○	○	○	○	+	+
4	○	○	○	○	○	○	○	○	○	○	+	-
5	○	○	○	○	○	○	○	●	○	○	+	-
6	○	○	○	○	○	×	○	○	○	○	+	-
7	○	○	○	○	○	○	○	×	○	○	+	-
8	○	○	○	○	○	○	○	○	○	○	+	+
9	○	○	○	○	○	○	○	○	○	○	+	-
10	○	○	○	○	○	○	○	○	○	○	+	-
11	○	○	●	○	×	●	×	○	●	●	+	-
14	○	●	○	○	○	○	●	○	○	○	+	+
15	○	○	○	○	○	○	○	○	○	○	+	-
18	○	○	○	○	○	○	○	○	○	○	-	-
19	○	○	○	○	○	○	○	○	○	○	+	-
20	○	○	○	○	○	○	○	○	○	○	-	-
22	○	●	○	○	○	○	○	○	○	○	+	-
24	○	●	○	○	○	○	○	○	○	○	-	-
27	○	○	○	○	○	○	○	○	○	○	+	-
29	○	●	○	○	○	○	○	○	○	○	-	-
30	×	○	○	○	○	○	○	○	○	○	+	-
31	○	○	○	○	○	○	○	○	○	○	-	-
32	○	○	○	○	○	○	○	○	○	○	-	+
33	○	○	○	○	○	○	○	○	○	○	+	+
34	○	○	○	○	○	○	○	○	○	○	+	-
35	○	○	○	○	○	○	○	○	○	○	+	-
36	×	○	○	○	○	×	○	○	○	○	+	-
37	○	○	○	○	×	●	×	×	○	○	-	-
38	●	○	○	○	×	●	○	○	○	○	+	-
39	●	○	○	○	○	×	○	○	○	○	+	-
40	○	○	○	○	○	●	○	○	○	○	+	-
41	×	○	○	○	○	○	○	○	○	○	+	-
42	×	○	○	●	○	×	●	○	●	●	+	-
43	○	○	○	○	○	×	○	○	○	○	+	-
44	○	○	○	○	○	○	○	○	○	○	+	+
45	○	●	○	○	○	○	○	●	○	●	-	-
47	○	○	○	○	○	○	○	○	○	○	+	-
48	×	○	○	○	○	×	×	○	○	○	+	-
49	×	○	○	×	○	×	○	●	○	○	-	+
50	○	○	○	○	○	○	○	○	○	○	+	-
51	●	○	○	○	○	○	○	○	○	○	+	+
52	×	○	○	○	○	○	○	○	○	○	+	-
53	○	○	○	○	○	○	○	○	○	○	-	+
54	○	○	○	○	○	○	○	○	○	○	+	-

Table 3. Genetic deletion for chromosome 3p in 43 cases with carcinoma of the cervix in relation to physical state of HPV

Locus	Heterozygosity	No. LOH	LOH frequency			
		(informative cases)	HPV 16[positive]	HPV 16 [negative]	HPV 18[positive]	HPV 18[negative]
D3S1038	0.84	3 (36)	3	0	1	2
D3S1110	1.00	5 (43)	2	3	1	4
D3S1029	1.00	1 (43)	1	0	0	1
D3S1076	0.98	1 (42)	1	0	0	1
D3S1295	0.93	0 (40)	0	0	0	0
D3S1592	0.81	4 (35)	3	1	0	4
D3S1313	0.93	2 (40)	2	0	1	1
D3S1228	0.95	4 (41)	1	3	1	3
D3S1287	1.00	2 (43)	2	0	0	2
D3S1284	1.00	3 (43)	3	0	0	3

○, marker showing retention; ●, marker showing LOH; ×, marker homozygous (not informative);
-, No HPV detected; +, HPV infection.

Discussion

PCR based microsatellite polymorphism analysis is a powerful tool in the study of genetic linkage and identification. LOH analysis are used to detect the chromosome deletions in tumor cell when the normal tissues have two different alleles. However, the sensitivity of the PCR test is strongly related to the size of the PCR product, especially in paraffin embedded tissues. In this study, the primers which amplified a sequence of less than 300 bp, were able to detect LOH and HPV infection in all 43 paraffin embedded cervical carcinomas. The purity of the cell population being analyzed is one of the most important factors in detection LOH. LOH can reliably be detected in tumor samples only if the content of tumor cells exceeds 70-80%. We used microdissection to enrich tumor cell although contamination of normal cells cannot be excluded completely. Recently laser capture technique has been developed to isolate individual cells from archival or fresh tissue.[9]

HPV infection has been suggested to play an important role in the development of cervical cancer. However, other genetic alterations seem to be required for the tumor development and progression because HPV infection does not always lead to cervical cancer. The chromosomal regions that are frequently lost are thought to harbor putative tumor suppressor.[10]

The 3p region has been implicated in ovarian cancer, breast cancer, testicular cancer, lung cancer and renal cell carcinoma, strongly suggesting the presence of a tumor suppressor gene in the region. Several different regions of allele loss were reported for chromosome 3p in cervical carcinomas, suggesting that relevant tumor suppressor genes reside at 3p24.1-ter, 3p21.1-24.2, 3p14.2-21.2, 3p14.1-14.2, and 3p11-12.1.[11] We fine-mapped a segment of 3p from 3p12 to 3pter and found that LOH was observed in fifteen of 43 tumor (35%). Other LOH studies have consequently resulted in a wide range of reported LOH frequencies of 35-100%. Herzog et al. (2001) detected a LOH on chromosome 3p in 7 of 15 cases with cervical cancer (47%) and found a common region of LOH at 3p14.1-25.[12] Wistuba et al. (1997) reported that 70% of cervical cancer showed LOH at 3p14.2-24.2,

and 3p14.2 (FHIT gene) (56%) and 3p21 (57%) were the most frequent 3p sites of loss.[13] Larson et al. (1997) found LOH at one or more contiguous loci in 55 of the 66 cases (83%).[11] Several studies agree that within the region 3p14-p22 two subregions where the LOH are concentrated are altered in the tumor.[14,15] Few studies have evaluated chromosome 3p abnormalities in CIN. Chung et al. (2000) reported 33% loss in 15 cases with low grade CIN (CIN 1), while 92% in 24 cases with high grade CIN (CIN 2 and 3).[16]

HPV types 16 and 18 are important causative agents in the etiology of cervical cancer.[17,18] The HPV-positive rates of 81% in our reports is in agreement with previously reported findings indicating that the association between HPV and cervical carcinoma is consistent worldwide. HPV prevalence in cervical cancer was 92.9% and ranged from 75-100%.[1]

We also evaluated the role of HPV infection in the deletion of the chromosome 3p but found no definite relationship. We would rather suggest that HPV infection does not change the genetic derivation of chromosome 3p. Fouret et al. (1998) showed that the relationship was not found between 3p13-21 loss of heterozygosity and HPV infection.[8] In the study of Su et al. (1998), no correlation between HPV infection and the abnormal FHIT transcript was identified.[19] Lu et al. (2000) reported that the correlation between HPV infection and alteration p53 or RB1 in cervical carcinomas has not been clearly confirmed by the in vivo evidence.[20] However, Mitra (1999) studied that the analysis of LOH frequencies in relation to HPV 16/18 infection revealed significant correlation in respect of 3p LOH sites.[21] There are numerous mechanisms which can result in tumor suppressor gene inactivation together LOH such as methylation, mutation or altered expression.

In summary, although HPV infection is probably the most important event, progressive deletions at one or more regions at 3p further supports the involvement of tumor suppressor genes in the pathogenesis of cervical carcinoma. Associations between the LOH of chromosome 3p and HPV infection were not significantly associated.

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■ 국문 초록 ■

자궁경부암 환자에서 염색체 단완의 유전자 변형과 HPV 감염

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임경택** · 성석주** · 박인서** · 심재욱** · 김태진**

자궁경부암에서 염색체 3번 단완의 유전자 변형이 자주 발생하며, 이것은 암의 성장에 큰 역할을 하는 암억제유전자가 염색체 3번 단완에 있을 것으로 추정되어졌다. 우리는 43예의 자궁경부암 환자의 슬라이드에서 암세포와 정상 상피세포를 추출하여 유전자 변형을 확인하였다. 3p12-26.1 부위에 존재하는 10개의 다형형질 마커를 갖고 polymerase chain reaction (PCR)을 실시하였다. 또한 자궁경부암의 발현요인으로 알려진 human papillomavirus (HPV) 16과 18의 감염여부도 PCR을 통해 확인하였다. 43예 중에서 15예만이 3번 염색체 단완의 하나 또는 여러 부위에서 유전자 변형을 보였다 (35%). HPV 16 또는 18의 감염을 보인 경우는 35예 (81%) 이며, HPV 16은 32예 (74%), HPV 18은 9예 (21%)로 확인 하였다. HPV 16에 감염된 32예 중 9예만이 유전자 변형을 보였고 (28%), HPV 16에 감염되지 않은 11예 중에서는 6예가 유전자 변형을 보였다 (55 %). HPV 18에 감염된 9예 중, 3예 (33%)만이 유전자 변형을 보였고, HPV 18에 감염되지 않은 34예 중 11예에서 유전자 변형을 보였다 (35%). 이는 염색체 3번의 유전자 변형과 HPV 감염과는 관계가 없는 것으로 확인되었다.

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