

# Clonality Analysis of Synchronous Lesions of Cervical Carcinoma Based on X Chromosome Inactivation Polymorphism, Human Papillomavirus Type 16 Genome Mutations, and Loss of Heterozygosity

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## Abstract

One of the most common forms of carcinoma in women, cervical invasive squamous cell carcinoma (CIC), often coexists with multiple lesions of cervical intraepithelial neoplasia (CIN). CIC and CIN show heterogeneity with respect to both histopathology and biology. To understand the causes, origin, and model of progression of cervical carcinoma, we assessed the clonality of a case with multiple synchronous lesions by analyzing X chromosome inactivation polymorphism, human papillomavirus type 16 (HPV16) sequence variation/mutations, and loss of heterozygosity (LOH). Microdissection was performed on 24 samples from this case, representing the entire lesional situation. The combination of different X chromosome inactivation patterns, two HPV16 point mutations, and LOH at three genomic microsatellite loci, led to the identification of five different “monoclonal” lesions (CIN II, CIN III, and invasive carcinoma nests) and five different “polyclonal” areas (CIN II and normal squamous epithelium). This finding indicated that CIC can originate from multiple precursor cells, from which some clones might progress via multiple steps, namely via CIN II and CIN III, whereas others might develop independently and possibly directly from the carcinoma precursor cells. Our results also supported the view that HPV16 as a “field factor” causes cervical carcinoma, which is probably promoted by the loss of chromosomal material as indicated by the LOH.

Key words: cervical carcinoma • X chromosome inactivation • HPV • LOH • clonality

## Introduction

Cervical invasive carcinoma (CIC)\* is one of the most common malignancies in females. Unlike in some other tumors, so far no specific oncogenes or tumor suppressor genes have been found in cervical carcinoma. Although certain specific types of human papillomavirus (HPV) have been regarded as the main cause of cervical squamous cell carcinoma (1–3), only a minority of HPV-infected cervixes develop any lesions, including cervical intraepithelial neoplasia (CIN) I, CIN II, and CIN III, and CIC (4). CIC commonly coexists with CINs in the same cervix. CIN lesions, which may regress, persist, or progress, are usually well demarcated from adjacent normal epithelium and have cells morphologically similar to CIC cells. Elimination of

CINs by surgical intervention reduces the occurrence of CIC (5, 6).

Consequently, it is assumed that CIC originates from a single founder cell and that the malignancy progresses by multiple evolutionary steps via CINs. If this is true, it could be a key to our understanding of the causes and mechanisms of carcinogenesis of cervical carcinoma and thus be very helpful in the design of rational prevention and treatment strategies. Clonality analysis has been confirmed to be a powerful tool with which to tackle this issue. If cervical carcinoma is monoclonal and the synchronous lesions show identical clonal patterns, this might favor the assumption that mutation of oncogenes or tumor suppressor genes is the cause of cervical carcinoma. Otherwise, it is more likely that cervical carcinoma is caused by some field factor such as HPV infection. Although several studies have put forward evidence supporting the monoclonal model of cervical neoplasia (7–9), there are a few reported cases of cervical carcinoma with contrary molecular features, i.e., signs of polyclonal origin (7, 8, 10).

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\*Abbreviations used in this paper: CIC, cervical invasive carcinoma; CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus; HPV16, human papillomavirus type 16; LOH, loss of heterozygosity.

The X chromosome inactivation pattern in a precursor cell is invariably inherited by subsequent descendants forming a cell lineage (11–13). The polymorphism of the X chromosome–linked androgen receptor gene, which consists of a short tandem repeat, [CAG] $n$  ( $n = 11 - 31$ ), has been widely used for clonality analysis of female tumors. However, interpretation of clonality information obtained from samples with the same and/or differing X chromosome inactivation patterns in an individual requires additional markers. HPV infection is believed to precede the initiation of cervical carcinoma and persists in almost all CIN and CIC lesions (1, 14). Human papillomavirus type 16 (HPV16) is the most commonly seen type of HPV in cervical squamous cell carcinoma (14) and sequence variations or mutations are frequent in HPV16 (15–19). If multiple cervical lesions in an individual patient have different HPV16 variants, this might indicate that they do not share a clonal origin. Thus, the HPV16 sequence can be one assistant clonality marker. Loss of heterozygosity (LOH) can be another as it occurs frequently in cervical carcinoma (20). Indeed, many clonality analyses based on LOH have been performed (10, 21).

To address the clonality of cervical carcinoma we selected one “golden” case for analysis instead of screening a large set of cases with statistical power. This case had many advantages: a CIC synchronous with CIN II and CIN III lesions; a moderate degree of differentiation so that it was possible to isolate carcinoma nests from normal tissue; separate carcinoma nests were available for easy microdissection; no conspicuous inflammatory cells infiltrating either the lesions or normal areas, which could interfere with X chromosome inactivation and LOH analyses; the patient had not undergone radiotherapy or chemotherapy before surgical extirpation; the entire cervix was available, from which we could take enough samples representing the whole set-up of cervical lesions observed; the sample was available as fresh tissue, which was preferable for restriction enzyme digestion and PCR; and the case was positive for HPV16 and informative for androgen receptor gene polymorphism and three of the screened LOH markers.

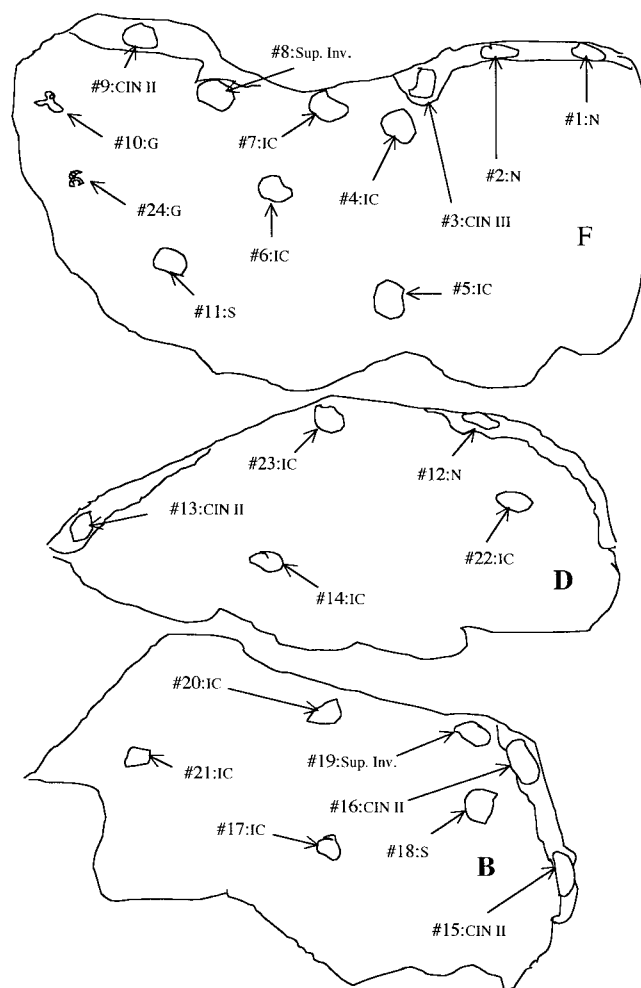
The main finding was that this case of cervical carcinoma was polyclonal. One of the invasive cancer clones could be traced back to its synchronous CIN II and CIN III lesions, whereas others had no specific intraepithelial precursors. This indicated that cervical carcinoma can originate from multiple precursor cells, from which some malignant clones might progress via multiple steps, namely CIN II and CIN III, whereas others might develop independently and possibly directly from the precursor cell. The results also strongly supported the opinion that HPV16 is the cause of cervical carcinoma.

## Materials and Methods

**Patient and Specimen.** Case H2 was a Swedish woman who had her uterus removed at the age of 33 because of cervical carcinoma. Macroscopically, the tumor grew within the cervix and around the external ostium without involving the uterus body or

vagina. The histopathological diagnosis made after microscopical examination was CIC (moderate differentiation) with invasion of local vessels and metastasis to local lymph nodes. 1 mo before the surgical procedure the patient had been found by vaginal cytology to have cervical malignancy. Subsequently this diagnosis had been confirmed by biopsy. HPV routine testing revealed HPV16 positivity. Before this HPV test, the HPV infectious situation was not known. At two vaginal cytological examinations 11 and 8 yr earlier no abnormality had been found. The entire fresh cervix was cut from the external ostium to the endocervix into six parts designated A, B, C, D, E, and F, in order. Parts A, C, and E were used for routine histopathological examinations, whereas B, D, and F were frozen at  $-80^{\circ}\text{C}$  for research.

**Microdissection.** 6  $\mu\text{m}$  of serial cryosections were prepared from parts B, D, and F, and stained briefly with Mayer’s hematoxylin. Multiple microdissections were performed on invasive cancer nests CIN II and CIN III, normal epithelium, and glands and stroma from different areas in a representative section for each tissue block. Altogether 24 samples (H2-1–24) were taken covering the whole lesional area. When it was necessary to repeat



**Figure 1.** Topography and histopathology of microdissected samples. Sides with F, D, or B (names of tissue block) are external ostium portions and the opposite sides are facing endocervix. #, H2-; arrows point to the foci where samples (named H2-1, -2, etc.) were microdissected; N, normal squamous epithelium; G, gland epithelium; S, stroma; IC, invasive carcinoma; Sup. Inv., superficially invasive carcinoma.

the microdissection procedure it was done using immediately adjacent sections that represented the same areas as originally chosen for sampling. Sample number, location, and morphology are summarized in Fig. 1. All invasive lesions were distinctly demarcated from stroma and CINs from adjacent normal epithelium (Fig. 2). Admixture of normal epithelial, stromal, or inflammatory cells was insignificant judging by careful examination under the microscope. Microdissection was performed with a scalpel, and the blade was changed after each microdissection. The microdissected pieces were transferred to Eppendorf tubes containing 50  $\mu$ l of PCR buffer II (PerkinElmer; Roche Molecular System). Each sample contained  $\sim$ 500–1,000 cells (22).

**DNA Preparation and Restriction Enzyme Digestion.** Lysis of cells overnight by 500  $\mu$ g/ml of proteinase K at 56°C was interrupted by incubation at 95°C for 10 min. 30  $\mu$ l of the 50  $\mu$ l of roughly prepared DNA from each sample was ready for use by PCR sequencing of HPV16 (23) and PCR gene scanning detection of LOH with microsatellite markers (24). The DNA in the remaining 20  $\mu$ l was further purified for X chromosome inactivation analysis (25). 50  $\mu$ l of 95% EtOH was added and the sample was incubated at  $-70^{\circ}$ C for 5 h. After centrifugation at 13,000

rpm for 30 min, the precipitate was washed with 250  $\mu$ l of 70% EtOH and spun at 13,000 rpm for 20 min and then air-dried. The pellet was dissolved in 20  $\mu$ l of reaction buffer for methylation-sensitive restriction enzyme HpaII digestion (Promega) and halved into two tubes. To one portion 15 U of HpaII was added and incubated overnight at 37°C. The reaction was terminated by heat inactivation at 95°C for 5 min. The other portion of DNA, as a control, was not exposed to HpaII but was otherwise treated in the same way. The non-HpaII-digested and HpaII-digested DNA portions were used for PCR amplification of the androgen receptor gene fragment.

**PCR.** Information on the sequences of PCR primers for the androgen receptor gene (two pairs), HPV16 genome (17 pairs), or human microsatellite DNA sequences (three pairs), as well as the magnesium concentration and the annealing temperature used for PCR with each primer pair are summarized in Table I. PCR was performed on a RoboCycler Gradient 96 (Stratagene).

Nested PCR was used to amplify the androgen receptor gene fragment (26). 20  $\mu$ l of reaction volume (PCR buffer II, 1.75 mM of MgCl<sub>2</sub>, 200  $\mu$ M of each deoxynucleotide, 0.5 U of Taq Gold DNA polymerase [PE; Roche Molecular System], 2 pmols

**Table I.** Sequences of PCR Primers and PCR Conditions

Names of PCR primers	Sense primer 5'-	Antisense primer 5'-	bp	[Mg <sup>2+</sup> ]	A.T.
				mM	°C
<b>X-ch. a.r.</b>					
P51/-54(outer)	gccgtccaagacctaccgaggagc	gctgtgaagggttgetgttctctcat	305	1.75	60
P52/-53(inner)	tccataatctgttccagagcgtgc	ggcttggggagaaccatcctcacc	226	1.75	61
<b>HPV16 (nt)</b>					
E6-1(24)/-2(646)	ctaaggggcgtaaaccgaaatc	tcctcctctgagetgtcatt	642	4.0	55
E7-1(505)/-2(892)	cgatgtatgtcttggttgcag	ttacattccgtaccctcttc	387	2.5	55
E1-1(816)/-6(1285)	catactaggaattgtgtgcc	acctgtaacatctgctgagt	489	2.5	55
E1-5(1285)/-2(1800)	actcagcagatgttacaggt	atgctgctgctgtactacgc	515	2.5	55
E1-3(1800)-8(2291)	cgtagtacagcagcagcatt	acctgtgttagctgcacat	511	2.0	55
E1-7(2291)/-4(2822)	atgggtgcagctaacacaggt	tggtcacgtaggctctgtact	551	2.5	55
E2-1(2703)/-2(3190)	tcaaggacgtgggtccagatt	acttgaccctctaccacagt	506	4.0	58
E4-1(3190)/-2(3689)	tgtggtagagggtcaagttaga	catgtagacgacactgcagta	538	2.5	55
E5-1(3710)/-2(4234)	ggcattggacaggacataat	cagaacgtttgtgtcgcatt	544	4.0	55
L2-1(3999)/-4(4479)	ataacagcagcctctgcgtt	ggagcaagtgtatctgtagc	500	4.0	58
L2-3(4479)/-2(5111)	gctacagatacacttgctcc	acgcctagagggttaatgctg	652	2.5	55
L1.2-1(5111)/L2-6(5639)	cagcattaacctctaggcgt	actaggcagccaaagagaca	548	2.5	55
L1-3(5639)/L1.2-2(6151)	tgtctctttggctgectagt	cattggtagatggggatcct	532	3.0	58
L1-1(6151)/-4(6601)	aggatccccatgtaccaatg	ggttaccaccaacaatgccca	470	3.0	58
L1-5(6601)/-2(7106)	tggcatttgggtgggtaacc	gtagaggtagatgagggtggt	525	2.5	55
LCR-1(7099)/-4(7528)	tacaccaccacctcatcta	tggcaagcaggaaacgtaca	449	2.5	58
LCR-3(7528)/-2(35)	tgtacgtttcctgcttgcca	tcggttcaaccgatttcggt	412	2.5	58
<b>Microsatellite markers</b>					
D3S659	ctgcaaggctctgtttaacag	attccagggacaagttcccc	105	1.5	55
D3S1283	ggcagtaccacctgtagaatg	gagtaacagaggcatcgtgtattc	156	1.5	55
D6S311	atgtcctcattgggtgtgtg	gattcagagcccaggaagat	237	1.5	55

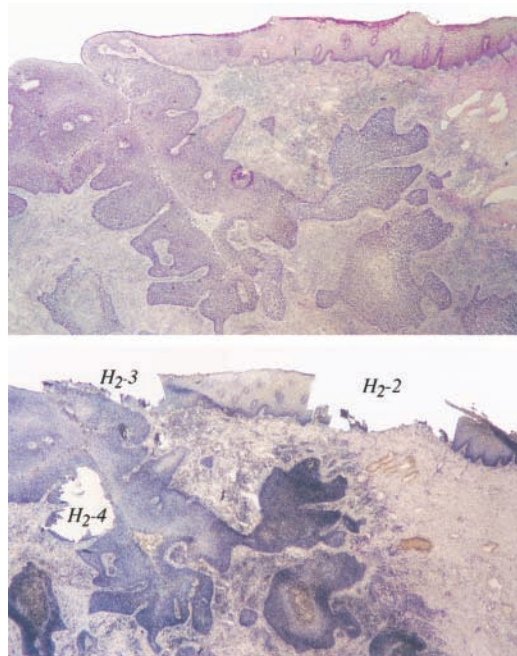
Sequences of PCR primers and PCR conditions. X-ch. a.r., inactivation polymorphism of X chromosome-linked androgen receptor gene; P51–54, primers for amplification of androgen receptor gene (reference 26); HPV16 (nt), initial nucleotide position of the primers used for amplification of the HPV16 genome including genes E1, E2, E4, E5, E6, E7, L1, L2, and LTR. We designed the primers according to the reference sequence (reference 30). The sequences of primers for the microsatellite markers were derived from The Genome Database (references 27–29). bp, length of PCR fragments; [Mg<sup>2+</sup>], concentration of MgCl<sub>2</sub>; A.T., annealing temperature.

of each sense and anti-sense primer, and 5  $\mu$ l of DNA solution with or without HpaII digestion) was used and 20 cycles (45 s at 95°C, 30 s at 60°C, and 1 min at 72°C) with 10 min initial denaturation at 95°C, and 7 min final elongation at 72°C were applied in the outer PCR. 2  $\mu$ l of the outer PCR product was then used for the inner PCR with a 20- $\mu$ l reaction mix containing the same concentration of PCR buffer II, MgCl<sub>2</sub>, each deoxynucleotide as listed above, 1 U of Taq Gold, and 5 pmols of inner primers. One of the inner primers was labeled in the 5' end with a fluorescent 6-FAM (Applied Biosystems) group to enable detection of the PCR products in the fragment analysis. We used 30 cycles with the same PCR program as described above, but at a higher annealing temperature of 61°C for the inner PCR.

To amplify the HPV16 genome, we used 30  $\mu$ l of reaction volume (PCR buffer II, MgCl<sub>2</sub> [at a concentration specific for each primer pair, shown in Table I], 200  $\mu$ M of each deoxynucleotide, 0.5 U of Taq DNA polymerase, 0.5  $\mu$ M of each sense and anti-sense primer, and 1  $\mu$ l of DNA solution). Thirty-five cycles (1 min at 95°C, 2 min at a temperature specific for each primer pair [shown in Table I], and 3 min at 72°C) with 10 min of initial denaturation at 95°C and 7 min of final elongation at 72°C were used.

To amplify microsatellite DNA, a 20- $\mu$ l mix containing the components at the same concentrations used for PCR, of HPV16, 35 cycles (30 s at 95°C, 45 s at 55°C, and 1 min at 72°C) with 10 min initial denaturation at 95°C, and 7 min final elongation at 72°C, was used. Amplification of D3S659 (3p13) (27), D3S1238 (3p24.2-3p22) (28), and D6S311 (6q22-6q23) (29) were chosen because these loci were informative in this case according to our pilot screening. PCR products were labeled by (R110) dUTP (PerkinElmer) during the PCR cycles.

To avoid contamination, we prepared a PCR master mix in an isolated room in a hood where UV light was used to destroy any potential contaminating DNA or PCR product at the working

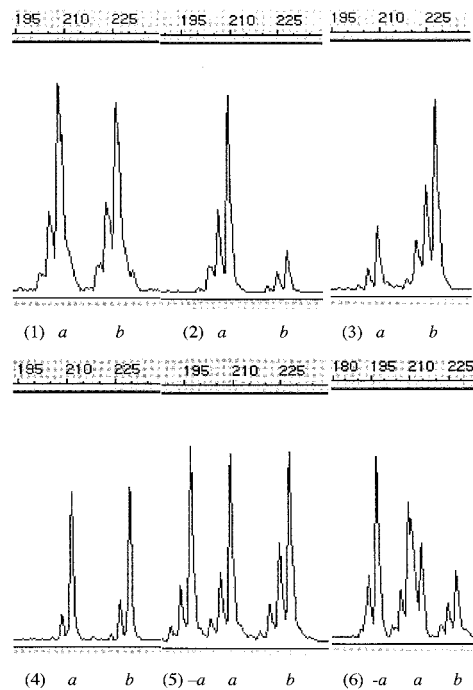


**Figure 2.** Representative photos illustrating the microdissection procedure. Upper, before microdissection; lower, after microdissection with the focus on spaces after the sampling of H2-2-4.

area before and after this manipulation. Template DNA was added under similar working conditions in a separate room.

**Fragment Analysis.** 1.5  $\mu$ l of the final PCR product of androgen receptor gene or a microsatellite locus was mixed with 0.5  $\mu$ l of loading buffer, 0.5  $\mu$ l of internal size standard GENE-SCAN-350 ROX (PerkinElmer), and 2.5  $\mu$ l of formamide, and denatured at 95°C for 5 min followed immediately by cooling on ice before loading on a 4.5% acrylamide gel. Electrophoresis was performed on an ABI Prism 377 sequencer (PerkinElmer). Size and quantity of allelic fragments and LOH were analyzed and determined by GeneScan Analysis 2.0.2 (PE; Applied Biosystems). A 60% or higher allelic signal reduction between any two alleles after HpaII digestion was considered a homogeneous pattern representing a sample with a paternally or maternally inactivated X chromosome (26). The same criterion of allelic reduction was adopted for judgement of LOH.

**Sequence Analysis.** HPV16 PCR amplicons were separated electrophoretically on 1.5% agarose gel and stained with ethidium bromide. Desired bands were cut out and subsequently purified on GenElute Minus EtBr Spin Columns (Supelco). The purified PCR products were quantified and then applied to enzymatic extension reactions for DNA sequencing using the Cycle Sequencing Ready Reaction Kit (Big-Dye terminator reagent containing dye-labeled terminators; PerkinElmer; Applied Biosystems) in



**Figure 3.** Electropherograms representative of different X chromosome inactivation patterns. (1) and (5), without HpaII digestion; (2)-(4) and (6), with HpaII digestion. (1), two alleles each with different times of a [CAG] tandem repeat, the short allele (defined as *a*) giving highest peak at 208 bp, a second peak at 205 bp, and a third peak at 202 bp, and the long allele (defined as *b*) is represented by highest peak at 226 bp, a second peak at 223 bp, and a third peak at 220 bp; (2), short allele remains, defined as *a* pattern (H2-3); (3), long allele remains, defined as *b* pattern (H2-7); (4), two alleles remain, defined as *ab* pattern (H2-8); (5), an extra allele (defined as *-a*) with highest peak at 193 bp, and a third peak at 190 bp; (6), *b* allele is reduced by 63% and *a* allele is reduced by 25% of *-a* allele, therefore this picture represents *-aab* pattern (H2-9). (5) and (6) represent a special pattern seen only in H2-9.

GeneAmp PCR Systems 9600 (PerkinElmer). The same forward and reverse primers used for the PCR amplification of HPV16 were used separately in cycle sequencing. The extension products were purified by ethanol/sodium acetate precipitation, and then electrophoresed on an ABI Prism 377 sequencer. The sequence and variations were analyzed and determined by the Factura™ and Sequence Navigator version 2.0 (PerkinElmer; Applied Biosystems). The test was repeated at least once for each sample starting from DNA-PCR amplification with the same result.

## Results

*X Chromosome Inactivation Patterns.* 23 out of 24 non-HpaII-digested samples showed two alleles (*a*, 208 bp; *b*,

226 bp) of the androgen receptor gene and one sample contained an additional allele (*-a*, 196 bp) (Fig. 3). The peaks for the alleles did not overlap in size, which made the H2 case ideal for analysis of polymorphism of the X chromosome inactivation-linked androgen receptor gene. After HpaII digestion, three common X chromosome inactivation patterns, *a*, *b*, and *ab*, were seen (Fig. 3). As shown in Table II, all three samples from normal epithelium, two CIN II samples, and one superficially invasive carcinoma sample had *ab*; one CIN II, one CIN III, one superficially invasive cancer sample, six invasive carcinoma samples, and all gland and stroma samples displayed *a*; and three invasive carcinoma samples displayed *b*. A CIN II

**Table II.** *X Chromosome Inactivation Pattern, HPV16 Sequence Variations and Mutations, and LOH of the Microdissected Samples*

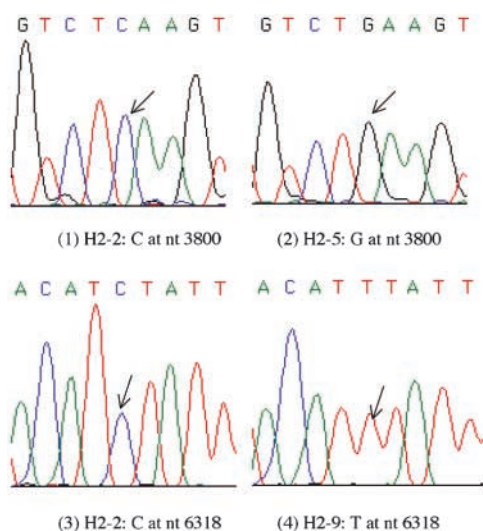
Sam. (H2-)	Histo- pathol.	Tissue block	Xc. pat.	HPV16 sequence variants					LOH			
				E2(nt 2926)	E2(nt 3800)	L2(nt 5226)	L1(nt 6318)	LCR(nt 7707)	V	D3S 659	D3S 1283	D6S 311
1	Sq. ep.	F	<i>ab</i>	g	g + c	c	—	g	V2	d	d	d
2	Sq. ep.	F	<i>ab</i>	g	—	c	—	g	V1	d	d	d
3	CIN III	F	<i>a</i>	g	—	c	—	g	V1	s	l	l
4	Inv. ca.	F	<i>b</i>	g	g	c	—	g	V2	s	l	l
5	Inv. ca.	F	<i>b</i>	g	g	c	—	g	V2	s	l	l
6	Inv. ca.	F	<i>a</i>	g	—	c	—	g	V1	s	l	l
7	Inv. ca.	F	<i>b</i>	g	—	c	—	g	V1	s	l	s
8	Sup. inv.	F	<i>ab</i>	g	—	c	—	g	V1	d	d	d
9	CIN II	F	<i>-aab</i>	g	—	c	t	g	V3	s	l	l
10	Gland	F	<i>a</i>	ng	ng	ng	ng	ng	ng	d	d	d
11	Stroma	F	<i>a</i>	ng	ng	ng	ng	ng	ng	d	d	d
12	Sq. ep.	D	<i>ab</i>	g	—	c	—	g	V1	d	d	d
13	CIN II	D	<i>a</i>	g	—	c	—	g	V1	s	l	l
14	Inv. ca.	D	<i>a</i>	g	—	c	—	g	V1	s	d	l
15	CIN II	B	<i>ab</i>	g	—	c	t	g	V3	s	l	l
16	CIN II	B	<i>ab</i>	g	—	c	t	g	V3	s	d	l
17	Inv. ca.	B	<i>a</i>	g	—	c	—	g	V1	s	l	l
18	Stroma	B	<i>a</i>	ng	ng	ng	ng	ng	ng	d	d	d
19	Sup. inv.	B	<i>a</i>	g	—	c	—	g	V1	s	d	l
20	Inv. ca.	B	<i>a</i>	g	—	c	—	g	V1	s	d	l
21	Inv. ca.	B	<i>a</i>	g	—	c	—	g	V1	s	l	l
22	Inv. ca.	D	<i>a</i>	g	—	c	—	g	V1	s	l	l
23	Inv. ca.	D	<i>a</i>	g	—	c	—	g	V1	d	l	l
24	Gland	F	<i>a</i>	ng	ng	ng	ng	ng	ng	d	d	d
HPV16 refer. seq. (reference 30)				a	c	a	c	a				
Amino acid change				q57q	q349e	l330f	s253f					

X chromosome inactivation pattern, HPV16 sequence variations and mutations, and LOH of the microdissected samples. Sq. ep., normal squamous epithelium; Sup. Inv., superficially invasive carcinoma; Xc. pat., X chromosome inactivation patterns (*a*, *b*, *ab*, *-aab*); —, the same nucleotide as the reference nucleotide at this position; ng, negative for HPV. q57q, the left q is the reference amino acid, the 57 is the codon position, and the right q is the amino acid resulting from the nucleotide shift found at this codon. Thus, q57q is a silent change, whereas the other three nucleotide changes are missense variations or mutations. V, HPV16 variant; s, short allele remains; l, long allele remains; d, both alleles remain and therefore normal.

sample (H2-9) was special. It seemed to have an extra allele. After HpaII digestion of this sample the two regular alleles were cut to different degrees but the additional allele could not be cut (Fig. 3). This fact indicated that although the emergence of the extra allele represented a true allele, it was not the result of contamination of a male or a homogeneous female DNA.

**DNA Sequence Variations and Mutations of the HPV16 Genome.** The entire genome of HPV16 with 7,905 bp was PCR sequenced for 20 out of 24 samples (the other four were HPV16 negative). Any nucleotide differing from that of the reference HPV16 sequence (30) is described here as a variation (probably occurring before infecting a specific host) or a mutation (probably occurring inside a specific host). Three variations and two mutations were found in this case (Table II). The first variation was a silent change from A to G within E2 (nt 2926). The second was a missense variation within L2 (nt 5226), in which nucleotide A was shifted to C (l330f). The last was a shift of A to G at nt 7707 within the long control region. The three variations were seen in all 20 HPV16-positive samples. The two mutations recorded were missense mutations and found only in a fraction of the samples. The first was at nt 3800 within E2 (Fig. 4), in which G replaced C (q349e). This change was present only in one sample from normal epithelium (H2-1) and in two invasive carcinoma (H2-4 and H2-5) samples. The second was a shift from C to T at nt 6318 within L1 (Fig. 4) (s253f), present only in three CIN II samples (H2-9, H2-15, and H2-16).

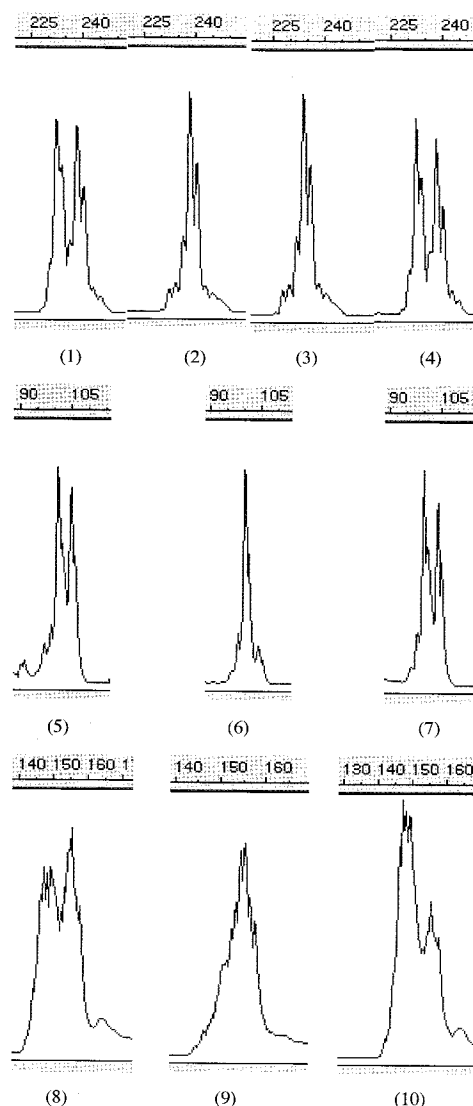
If an HPV16 “variant” is defined as an HPV16 genome with at least one different nucleotide within the whole sequence (22, 30), three HPV16 variants (V1–3) were found (Table II). V1 was the variant with the three common sequence variations as described above and was distributed



**Figure 4.** Electropherograms showing missense mutations of the HPV16 sequence. (1) and (3), reference sequences of HPV16 E2 (H2-2) and L1 (H2-2), respectively; (2), G replaced C at nt 3800 within E2 (H2-5); (4), T replaced C at nt 6318 within L1 (H2-9).

among most of the samples; V2 had the three sequence variations in addition to the mutation in E2 (nt 3800); and V3 had the three sequence variations in addition to the mutation in L1 (nt 6318).

**LOH.** Analysis was successful and informative for D3S659 (101 bp, 105 bp), D3S1283 (150 bp, 156 bp), and D6S311 (231 bp, 237 bp) in all samples (Fig. 5). Loss of the long allele for D3S659 and of the short allele for D3S1283 occurred. Most samples with LOH at D6S311 had lost the short allele, whereas one had lost the long allele. None of the normal samples, squamous epithelium, gland and stroma, nor the superficially invasive carcinoma sample (H2-8), showed loss at any of these three microsatellite



**Figure 5.** Electropherograms showing LOH analyzed at three different microsatellite loci. (1), (5), and (8) are normal tissue (H2-2), and the others are CIN or invasive carcinoma samples. (1)–(4), D6S311 (231 bp, 237 bp); (2), long allele remains (H2-3); (3), short allele remains (H2-7); (4), both alleles remain (H2-8); (5)–(7), D3S659 (101 bp, 105 bp); (6), short allele remains (H2-3); (7), both alleles remain (H2-8); (8)–(10), D3S1283 (149 bp, 156 bp); (9), long allele remains (H2-3); (10), both alleles remain (H2-8).

markers. The detailed information showing that LOH occurred in most lesion samples is given in Table II.

**Clonality Status Defined by Combined Analysis of Three Clonality Markers.** The carcinoma cell populations with different X chromosome inactivation patterns were definitely induced from cells with different clonality status. However, samples with identical X chromosome inactivation patterns could not be proven in this way to share the clonality status. HPV16 variants and LOH markers helped us to identify the clonality status of the samples having identical X chromosome inactivation patterns. As shown in Fig. 6, altogether four different monoclonal families (*a1*, *a2*, *a3*, *b6*, and *b7*) and five different polyclonal tribes (*a6b1*, *a8b3*, *a9b4*, and *-aa10b5*) were determined by the combination of the three markers. One sample of morphologically normal squamous epithelium with HPV infection represented an additional polyclonal tribe (*a7b2*). The samples from two normal glandular and two stromal areas contained the *a* pattern and were negative for both HPV16 infection and LOH.

**Plane Topography of the Different Clonal Lesions.** The plane topography of the different clonal lesions is illustrated by schematic drawings of sections from the tissue blocks (Fig. 7). When observed in two dimensions some members with the same clonal status could be seen close to each other, whereas others were further away from each other.

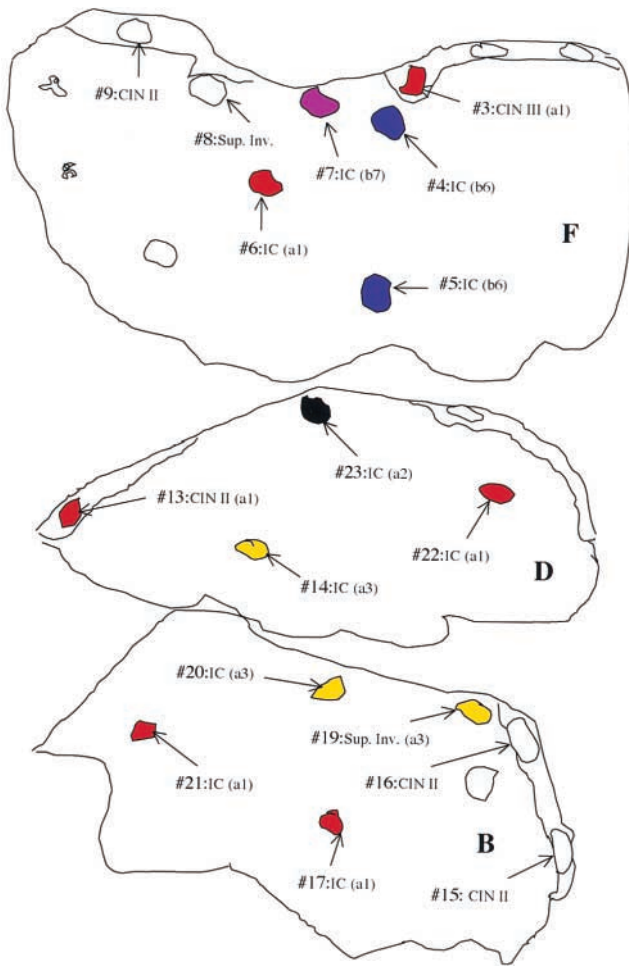
## Discussion

The results clearly indicated that this case of cervical carcinoma originated from multiple precursor cells, and that the process of carcinogenesis could take either multiple steps via CINs, or develop independently and directly into carcinoma from normal precursors. The results also indicated that HPV16 was the direct cause of this cervical carcinoma because HPV, as the field factor, must have been present in multiple target cells (defined by their X chromosome inactivation pattern) that not only yielded different clones of cervical carcinoma but also morphologically normal epithelium. When abnormally stimulated, as by HPV infection, and reinforced as by loss of crucial tumor suppressor genes, local stem cells might become tumor precursor cells (31) from which the neoplasm develops. The pattern of X chromosome inactivation in addition to the HPV16 mutations and the LOH at the three genomic loci, were regarded as a reflection of the clonality status of the respective samples. With this information at hand the derivation of the samples from different precursor cells could be deduced.

Two neighboring normal glandular areas and two separate samples of stroma, all of which showed the *a* pattern of X chromosome inactivation, might not represent a “monoclonal” origin, but rather a skewed distribution of the progenitor cells with the *a* pattern of X chromosome inactivation in the normal mosaic (32). Three areas of normal

Normal precursor cells (Xc. patterns)	<i>a</i>	<i>a</i>	<i>a</i>	<i>a</i>	<i>a</i>	<i>ab</i>	<i>ab</i>	<i>ab</i>	<i>ab</i>	<i>-aab</i>	<i>b</i>	<i>b</i>
HPV16 infec.	+	+	+	-	-	+	+	+	+	+	+	+
HPV16 V	v1	v1	v1	-	-	v1	v2	v3	v3	v3	v2	v1
D6S311 LOH	+	+	+	-	-	-	-	+	+	+	+	+
D3S659 LOH	+	-	+	-	-	-	-	+	+	+	+	+
D3S1283 LOH	+	+	-	-	-	-	-	+	-	+	+	+
Precursor cell subgroups	<i>a1</i>	<i>a2</i>	<i>a3</i>	<i>a4</i>	<i>a5</i>	<i>a6b1</i>	<i>a7b2</i>	<i>a8b3</i>	<i>a9b4</i>	<i>-aa10b5</i>	<i>b6</i>	<i>b7</i>
Normal	↓	↓	↓	↓ #10 #24 (g)	↓ #11 #18 (s)	↓ #2 #12 (sq)	↓ #1 (sq)	↓	↓	↓	↓	↓
CIN II	#13	↓	↓					#15	#16	#9		
CIN III	#3	↓	↓									
Inv. Ca.	#6 #17 #21 #22	#23	#19 #14 #20			#8					#4 #5	#7

**Figure 6.** Chart of clonality status. Xc. patterns, X chromosome inactivation patterns; *a* or *b*, represents the X chromosome-inactivated allele(s) of the androgen receptor gene; dashes or lines, indicate the suggested order in which the different events have occurred; arrows, symbolize that the HPV16 mutants were supposed to be derived from the V1 variant, and that the lesions or normal samples originated from different precursor cells; V, HPV16 variants; +, positive for HPV16 or LOH; -, negative for HPV16 or LOH. +\*, loss of another allele compared with the common one from the other samples in this case; #, H2-; (g), gland; (s), stroma; (sq), normal squamous epithelium.



**Figure 7.** Plane topography of the different clonal lesions. #, samples (H2-); a1, a2, a3, b6, or b7, the names of different clonal families. Samples with same color share the clonality patterns. Red, a1 (a,v1,s,l,l, in the order of X chromosome inactivation pattern; HPV16 variant; LOH pattern at D6S311; LOH pattern at D3S659; and LOH pattern at D3S1283); black, a2 (a,v1,d,l,l); yellow, a3 (a,v1,s,d,l); blue, b6 (b,v2,s,l,l); violet, b7 (b,v1,s,l,s). The normal samples are not given labels and the polyclonal lesion samples are not given color.

squamous epithelium displayed polyclonal patterns indicating that the squamous epithelium of this case was a fine mosaic of cell clones.

In this case all invasive carcinoma nests but one showed a monoclonal X chromosome inactivation pattern (a or b). The one (H2-8) with ab pattern might have been contaminated by normal epithelial cells when microdissection was performed, as the dissected area was a superficial cancer nest adjacent to normal cells. Unlike all CINs and 11 out of 12 carcinoma samples, neither H2-8 nor any of the normal samples showed allelic loss at any of the three loci observed. This result further supports the assumption that H2-8 had become contaminated with normal cells.

One interesting CIN II sample, H2-9, had the -aab pattern. The extra -a allele was probably produced by microsatellite instability in the early stage (CIN II) of cervical carcinoma and appeared not to affect the carcinogenesis as

it seemed to be limited to only one of the CIN II lesions. This sample was completely different from all other samples in its clonality pattern, which in addition to the finding of two different polyclonal CIN II samples, reinforces the conclusion that this case of cervical carcinoma was of polyclonal origin.

The HPV16 positivity in all lesions and squamous epithelium samples in this case indicated that the HPV infection had occurred before the initiation of any lesion. HPV16 is known to infect squamous cells specifically (33). Consistent with this, the glandular and stromae samples in this case were HPV16 negative. The two nucleotide changes observed in V2 (nt 3800) and V3 (nt 6318), respectively, might be very rare in the infectious HPV16 pool as they have not yet been reported by others. Moreover, the changes were additional to those in V1 and each appeared independently in a small proportion of the samples. Therefore, they were regarded as mutations. Most reported cases of cervical carcinoma have contained both episomal and integrated HPV16 (34–37). The case analyzed here probably also contained both episomal and integrated HPV16. With a pair of “back-back” primers in L1 of HPV16 and “long-PCR” technique, amplicons of 7.9 kb (indicating the complete circle episomal HPV16 genome), 2 kb, and 0.6 kb (representing at least three copies of HPV16 joined together in a tail to head fashion with numerous intermediate sequence deletions, which can occur in an integrated form of HPV16) were obtained in this case (H2) (unpublished data). Each integrated HPV16 variant in precursor cells can be passed down to the progeny in a single copy by cell division, whereas the episomal form of an HPV16 variant will be replicated to yield many copies that are divided between the two daughter cells. HPV residing in undifferentiated cells, such as tumor cells or tumor precursor cells, usually cannot produce complete infectious viral particles capable of infecting neighboring cells because the capsid proteins, L1 and L2, of HPV cannot be fully expressed in undifferentiated cells (38). So, if an HPV mutation occurs in (or a different HPV variant infects) tumor precursor cells, it might be passed down specifically to the progeny and remain long enough to become detected. Based on this assumption, the HPV16 variants were used here as assistant clonality markers.

LOH occurs quite often in cervical carcinoma (39). At some loci it starts in an early stage of the carcinogenic process and is nonrandom in nature (40). The three markers used in this study evidenced LOH in all CIN II and CIN III samples from this case, which suggested that the losses at these loci were early events. They helped us to divide or prove the clonality status of the samples determined by X chromosome inactivation patterns and HPV16 variants.

Since all of the clonality markers used appeared during an early stage of the carcinogenesis (in morphologically normal epithelium or CINs) as described above, the clonality information obtained could merely be a reflection of the clonality of precursor cells. To what degree the results reflect the development of subclones occurring in the evolution of cervical carcinoma is not known. To elucidate this



exhaustively, a control study such as the investigation of multiple subclones from a single tissue culture of an HPV-related tumor, can be done to determine if the clonality, allelic assays, and HPV sequence information were uniform in a monoclonal system over time.

Both monoclonality (41, 42) and polyclonality (7, 10) of tumors, including cervical carcinoma, have been reported, although most published data suggest the monoclonal model. Even though our results support the polyclonal origin of cervical squamous cell carcinoma, they could not exclude the possibility that a proportion of cervical carcinoma cases are monoclonal. To elucidate this issue, detailed analysis of more cases with synchronous lesions is necessary. Two recently published articles that deal with the subject of instability of X chromosome methylation in aberrant crypt foci of human colon and in lung cancer cells should also be noted (43, 44). It is not yet known if this phenomenon occurs in tumors and if it can affect the appropriate methylation of the X chromosome-linked androgen receptor gene in cervical carcinoma.

The members of each clonal lesion in this case were not always located close to each other in the plane of the section (Fig. 7). This does not necessarily indicate a lack of topographical relation between clonal members, although a topography in three dimensions needs to be constructed to address this issue. However, this goes beyond the aim of the present work.

In summary, our findings of polyclonality of cervical carcinoma support the opinion that HPV16 infection is the cause of cervical carcinoma. A conceivable scheme for the carcinogenesis of cervical carcinoma is that HPV16 infects precursor cells at an appropriate location such as the transformation zone and/or in a suitable epigenetic situation. Those cells might then acquire LOH and develop into independent CINs or directly initiate CIC. For the CIN lesions some unclear additional factors would decide their fate: to regress, to persist, or to progress. Those HPV16-infected precursor cells that lack propitious conditions for carcinogenesis would still give rise to normal epithelium, which may explain why only a minority of HPV16-positive cases develop any cervical lesion, and why in a case of cervical carcinoma with synchronous lesions at different stages, some of the normal squamous epithelium can be HPV16 positive, as are the lesions.

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