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Serum Antibody against Unfused Recombinant E7 Protein of Human Papillomavirus Type 16 in Cervical Cancer Patients

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Sera were examined for the presence of antibody against E7 protein of human papillomavirus type 16 (HPV-16) by Western blot analysis using the bacterially derived unfused protein. The occurrence rates of anti-E7 antibody against HPV-16 were 14.1% (10/71) in cervical cancer patients, 0% (0/48) in cervical intraepithelial neoplasia patients, and 0% (0/41) in female non-malignant patients. Three patients (one with endometrial cancer, one with breast cancer, and one male patient with colon polyp) out of 115 patients with tumors in organs other than the cervix, had antibody against E7 protein of HPV-16. The serum antibody, once positive, could be detected for a long time after surgical removal of the cancers in all cases that could be followed up. HPV-16 DNA could be detected in 50% (13/26) of cervical cancer patients. Sixty-nine percent (9/13) of patients with HPV-16 DNA in cancers had the antibody and all the patients with stages II, III, and IV cervical cancer (8/8) harboring HPV-16 DNA showed the presence of the antibody against E7 protein of HPV-16. In contrast, only 20% (1/5) of cervical cancer patients with stage Ia or Ib harboring HPV-16 DNA showed positive for the anti-E7 antibody in sera. These findings suggest that the presence of anti-E7 antibody in serum depends on the staging of cervical cancer and extent of HPV infection.

Key words: Human papillomavirus — Antibody — Cervical cancer

Epidemiological data have long suggested that a venereally transmitted infectious agent causes cervical neoplasia. Specific types of human papillomavirus (HPV), including HPV types 16 (HPV-16), 18, 31, 33, 35, 52 and 58, have been detected in cervical cancer tissues or cell lines. In particular, HPV-16 is present in about 50% of cervical cancer tissues.[1,2]

In vitro, HPV-16 or -18 DNA has the ability to transform established rodent cell lines[3] and to immortalize primary rodent or human keratinocytes.[4,5] Thus, HPV may play an important role in the development of cervical cancers. E6 and E7 open reading frames (ORFs) are consistently retained and expressed in cervical cancers with HPV DNA.[6–8] Recent studies have demonstrated that the E7 protein has transforming ability[9] and can form complexes with the retinoblastoma-associated tumor suppressor gene product.[10,11]

Despite the accumulating knowledge about the events necessary for cellular transformation, little is known about the immune response against HPV infections. In previous studies, we reported the successful production of the unfused HPV-16 E7 protein in *Escherichia coli* and the purification of the protein to near-homogeneity.[12] We used this protein as an antigen to detect the anti-E7 antibody in human sera by Western blot analysis. Here we report that all the patients examined with stages II, III, and IV cervical cancer harboring HPV-16 DNA had the anti-E7 antibody in their sera, while only one out of five patients with stage Ib possessed the antibody. We also report that the antibody, once detected, remained positive in sera for at least 2 years after total hysterectomy.

MATERIALS AND METHODS

Human sera Human sera were obtained from 234 patients attending the National Cancer Center Hospital, Keio University Hospital, and Shizuoka Red Cross Hospital from 1990 till 1992. Patients were classified into four groups: Groups A, B, C, and D. Group A consisted of 71 cervical cancer patients; stage I (n=34), stage II (n=24), stage III (n=9) and stage IV (n=4). Sixty-eight sera were drawn at the time when the cancer was discovered or during the first treatment. The other 3 had been obtained from patients treated about 10 years ago; 2 patients had had no recurrence, while one had shown recurrence at the time the serum was obtained. Group B consisted of 48 cervical intraepithelial neoplasia (CIN) patients. Group C comprised 64 other cancer patients, consisting of 49 females and 15 males. Group D consisted of 51 patients without cancer (41 females and 10 males). All sera were stored at −20°C until they were used.

Western blot analysis The E7 purified protein we had reported previously[12] was subjected to gel electrophoresis (5 ng per lane) on 5% stacking/15% separating

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sodium dodecyl sulfate (SDS)-polyacrylamide gels at 30 mA current. After electrophoresis, the protein was electroblotted onto Immobilon-P membranes (Millipore) at 0.8 mA/cm^2 for 1.5 h using a semi-dry blotting apparatus (Bio-Rad). For Western blot analysis, filters were cut into 2 mm strips. The strips were blocked at 4°C for 12 h with blocking mixture containing 4% nonfat dried milk, 25 m*M* Tris-HCl (pH 8.0), 125 m*M* NaCl, 0.1% Tween 20, 0.1% NaN_3. They were incubated with sera diluted 1:50 in blocking mixture at 4°C for 12 h, and washed three times with the blocking mixture. Then the membranes were incubated at room temperature for 1 h with alkaline phosphatase-conjugated goat anti-rabbit or anti-human IgG (Cappel) diluted 1:2000 in blocking mixture. After being washed twice with the blocking mixture and three times with 25 m*M* Tris-HCl (pH 8.0), 125 m*M* NaCl and 0.025% Tween 20, the membranes were stained with 0.4 m*M* Nitro Blue Tetrazolium and 0.4 m*M* 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt in 0.1 *M* Tris-HCl (pH 9.5), 0.1 *M* NaCl and 5 m*M* $MgCl_2$.

DNA extraction Total cellular DNA from frozen tissue specimens was extracted by SDS-proteinase K treatment, followed by phenol extractions. DNA from formalin-fixed, paraffin-embedded tissue blocks from patients was extracted as described previously[13] with the following modification. A single 50 μm section cut from the block was placed in an Eppendorf tube. The sections were deparaffinized in 1.5 ml of xylene, then centrifuged at 15,000 rpm for 10 min at room temperature, and the xylene was removed by decantation. The residual xylene was washed out with ethanol. The tissue pellets were dried, lysed with 400 μl of lysis buffer (1% SDS, 2 m*M* EDTA, 20 m*M* Tris-HCl, pH 7.4) and 5 μl of proteinase K (10 mg/ml) and incubated at 48°C, followed by extraction with phenol-chloroform. DNAs were precipitated with ethanol and dissolved in 10 m*M* Tris-HCl (pH 8.0), and 1 m*M* EDTA.

Detection of HPV DNA Two types of polymerase chain reaction (PCR) detection system were employed. In order to identify HPV-16, an HPV detection system with type-specific primers and an oligonucleotide probe was used as described.[14] Primers used were p16-1 (5′-AAGGGCGTAACCGAAATCGGT-3′), p16-2R (5′-GTTTGCAGCTCTGTGCATA-3′), and p33-2R (5′-GTCTCCAATGCTTGGCACA-3′). The oligonucleotide probe used was pB16-I (5′-CATTTTATGCACCAAAAGAGAACTGCAATG-3′). After amplification by PCR, 10 μl (1/10 volume) of the reaction mixture was subjected to gel electrophoresis on a 3.5% agarose gel and stained with ethidium bromide. DNA was transferred from the

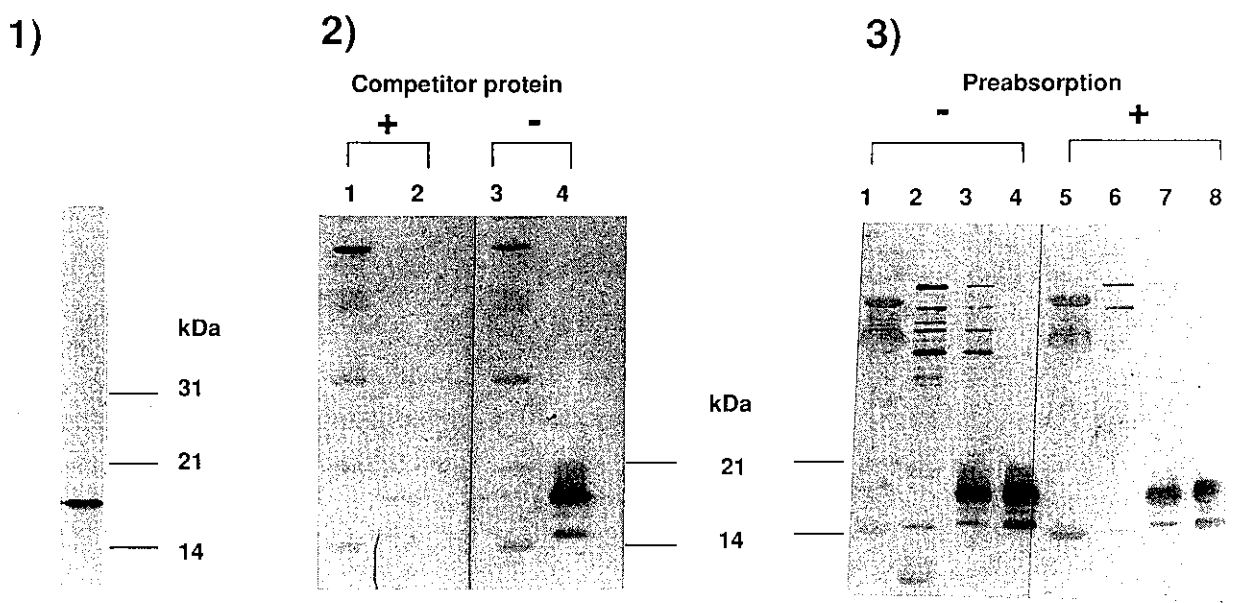

Fig. 1. Specificity of Western blot analysis for detection of the anti-E7 antibody in sera. 1) Five ng of E7 protein was subjected to gel electrophoresis and affinity-purified anti-E7 rabbit serum (prepared in our laboratory) was used in Western blot analysis. 2) E7 competition assay. One μg of marker protein (Bio-Rad) in Lanes 1 and 3, or 5 ng of E7 protein in lanes 2 and 4 was subjected to the gel electrophoresis. Proteins were transferred to the membrane filter, and incubated with 1:50 diluted serum from patient No. 5 pre-absorbed with E7 protein as a competitor (lanes 1 and 2) or not pre-absorbed (lanes 3 and 4). 3) *E. coli* absorption test. The proteins were subjected to gel electrophoresis. After blotting, they were incubated with the same serum (No. 5) pre-absorbed with *E. coli* lysate (lanes 5, 6, 7, and 8) or not (lanes 1, 2, 3, and 4); lanes 1 and 5 were marker proteins, lanes 2 and 6 were *E. coli* lysate, lanes 3 and 7 were *E. coli* lysate harboring E7 protein, and lanes 4 and 8 were E7 protein (50 ng).

gel to a Hybond N+ (Amersham) membrane filter. After prehybridization for more than 2 h, hybridization was performed at 42°C for 8–12 h in a hybridization solution (30% formamide, 0.1 *M* PIPES-NaOH pH 6.8, 0.65 *M* NaCl, 5×Denhardt's, 5 m*M* EDTA, 0.1% SDS, 10% dextran sulfate, 100 μg/ml denatured salmon testis DNA), and ^{32}P-end-labeled HPV-specific oligonucleotide probes at 2×10^5 cpm/ml. After hybridization, the filters were washed twice in 0.2×SSC, 0.06% sodium pyrophosphate, 20 m*M* sodium phosphate buffer (pH 7.0) and 0.1% SDS at room temperature for 15 min twice and at 50°C twice for 30 min. The filters were exposed to Kodak X-OMAT AR film at −70°C for 1 to 4 days.

When HPV-16 DNA could not be detected using the method described above, consensus primers for the L1 region were used under the reported PCR conditions.[15,16] Briefly, 100 ng of cellular DNA was amplified with L1C1 (5′-CGTAAACGTTTTCCCTATTTTTTT-3′) and modified L1C2 (5′-TACCCTAAATACCCTA-TATTG-3′) primers. HPV-DNA was typed on the basis of the restriction map analysis of the amplified HPV fragments. In the case of unknown types of HPV, an aliquot of the L1 PCR products was cloned into PCR™-II using the TA cloning kit (Invitrogen) or into lambda zap II (Stratagene) and then sequenced using the dideoxynucleotide chain-termination method.

Table I. Occurrence of the Anti-E7 Antibody against HPV-16

Group	Diagnosis	Female	Male	Total
A	Cervical cancer	14.1% (10/71)		14.1% (10/71)
B	CIN	0% (0/48)		0.0% (0/48)
C	Other cancer	4% (2/49)	0% (0/15)	3.1% (2/64)
D	Non-malignant disease	0% (0/41)	10% (1/10)	2.0% (1/51)

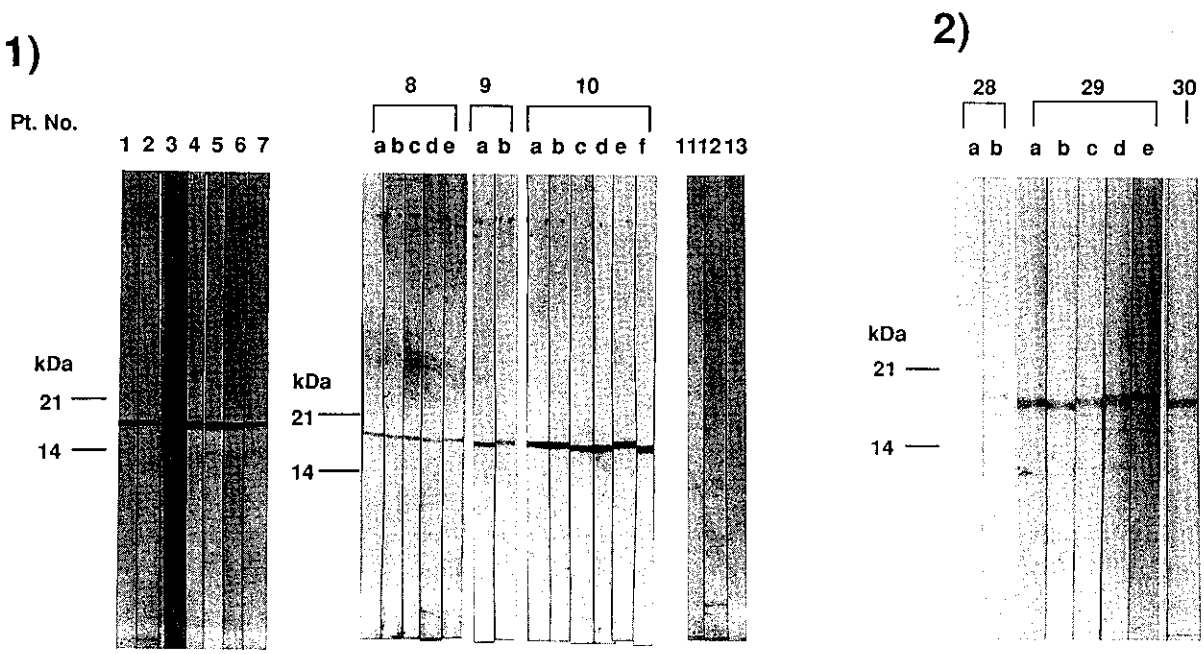

Fig. 2. Detection of the IgG antibody against HPV-16 E7 protein by Western blot analysis. The unfused E7 protein was subjected to gel electrophoresis on SDS-polyacrylamide gels and transferred to membranes. Strips were incubated with sera diluted 1:50. The numbers on the lanes indicate the patient numbers in Table II. 1) Sera from cervical cancer patients were examined. Lane 8a was before treatment, and 8b, 8c, 8d, and 8e were 1, 3, 12, and 16 months after treatment, respectively. Lane 9a was before treatment, and lane 9b was 12 months after treatment. Lane 10a was before treatment, and lanes 10b, 10c, 10d, 10e, and 10f were 1, 3, 5, 14, and 15 months after treatment, respectively. 2) Sera from other cancer patients and a colon polyp patient. Lanes 28 were endometrial cancer sera. Lane 28a was before treatment, 28b was 12 months after treatment, respectively. Lanes 29 were breast cancer sera. Lanes 29a, 29b, 29c, 29d, and 29e are 7, 13, 17, 23, and 29 months after mastectomy, respectively. Lane 30 was colon polyp serum.

RESULTS

Western blot analysis of the anti-E7 antibody in human sera The unfused E7 protein used in Western blot analysis had been produced in *E. coli* and purified to near-homogeneity (Fig. 1-1). In order to check the specificity of the reaction, a competition experiment and *E. coli* absorption test were done. The anti-E7 antibody reactivity of serum from patient No. 5 (Table II) was reduced after absorption with purified E7 protein (Fig. 1-2) and the antibody reactivity was unaffected by pre-absorption with *E. coli* lysate (Fig. 1-3).

Two hundred and thirty-four sera were tested for the presence of the anti-E7 antibody by Western blot analysis. The anti-E7 positive samples always gave positive reactions, while the negative ones gave negative results, reproducibly. Among female patients, 14.1% (10/71) of sera from cervical cancer patients (group A), 0% (0/48)

Table II. Detection of the Anti-E7 Antibody and HPV DNA Sequences

Group[a]	Patient No.[b]	Age	Diagnosis	FIGO[c]	TNM[d]	Recurrence	Western blot	Tissue source[e]	HPV-16 DNA[f]	HPV type[g]	L1 partial sequence
A	8	32	Cervical cancer	Ib	pT1bN0M0	(−)	(+)	F	(+)	16	ND
	9	58	Cervical cancer	Ib	pT1bN0M0	(−)	(+)	F	(−)	31	ND
	10	56	Cervical cancer	IIa	pT2bN1M0	(+)	(+)	F	(+)	16	ND
	7	56	Cervical cancer	IIa	pT2aN0M0	(−)	(+)	P	(+)	16	ND
	1	42	Cervical cancer	IIb	T2bNXM0	(+)	(+)	P	(+)	ND	ND
	2	48	Cervical cancer	IIb	pT2aN0M0	(−)	(+)	F	(+)	(−)	ND
	3	64	Cervical cancer	IIIa	T3aNXM0	(?)	(+)	P	(+)	ND	ND
	5	68	Cervical cancer	IIIb	T3bNXM0	(+)	(+)	P	(+)	16	ND
	6	59	Cervical cancer	IIIb	T3bNXM0	(+)	(+)	P	(+)	16	ND
	4	58	Cervical cancer	IV	T3bN1M1	(+)	(+)	P	(+)	ND	ND
B	27	43	CIN			(−)	(−)	F	(−)	58	ND
A	11	50	Cervical cancer	Ib	pT1bN0M0	(−)	(−)	F	(−)	(+)	X[i]
	12	48	Cervical cancer	Ib	pT1bN0M0	(−)	(−)	P	(+)	ND	ND
	13	54	Cervical cancer	Ib	pT1bN0M0	(−)	(−)	F	(−)	18	ND
	14	65	Cervical cancer	Ib	pT1bN0M0	(?)	(−)	F	(−)	(+)	ND
	15	40	Cervical cancer	Ib	pT1bN0M0	(−)	(−)	F	(−)	31	ND
	16	73	Cervical cancer	Ib	pT1bN0M0	(−)	(−)	P	(+)	(+)	16
	17	39	Cervical cancer	Ib	pT1bN1M0	(−)	(−)	F	(+)	16	ND
	18	55	Cervical cancer	Ib	pT2bN1M0	(−)	(−)	F	(33+)[h]	33	ND
	19	49	Cervical cancer	Ib	pT1bN0M0	(−)	(−)	F	(−)	(+)	X
	20	36	Cervical cancer	Ib	pT1bN0M0	(−)	(−)	P	(−)	52	ND
	21	33	Cervical cancer	Ib	pT1bN0M0	(−)	(−)	P	(+)	(+)	16
	22	53	Cervical cancer	Ib	pT1bN0M0	(−)	(−)	P	(−)	(+)	58
	23	55	Cervical cancer	IIa	pT2aN1M0	(+)	(−)	F	(−)	18	ND
	24	53	Cervical cancer	IIb	pT1bN1M0	(−)	(−)	F	(−)	52	ND
	25	63	Cervical cancer	IIb	pT2bN0M0	(−)	(−)	F	(−)	(−)	ND
	26	52	Cervical cancer	IIb	pT1bN0M0	(+)	(−)	P	(−)	58	ND
C	28	64	Endometrial cancer				(+)	(−)	ND	ND	ND
	29	70	Breast cancer				(+)	(−)	ND	ND	ND
D	30	64	Colon polyps				(+)	P	(−)	(+)	16, 33

a) Groups A–D, see "Materials and Methods."
b) The patient number matches that in Fig. 2.
c) Clinical staging was assessed according to the Fèdèration Internationale de Gynecologie et d'Obstetrique.
d) Pretreatment clinical classification (TNM classification) or post-surgical histopathological classification (pTNM) describes the extent of the primary tumor, the condition of lymph nodes, and distant metastasis, respectively. N0, no evidence of regional lymph node involvement; N1, evidence of involvement of pelvic lymph nodes; NX, regional lymph nodes could not be assessed; M0, no evidence of distant metastasis; M1, evidence of distant metastasis.
e) F; frozen tissues. P; formalin-fixed, paraffin-embedded tissues.
f) HPV DNA was detected with type-specific primers (p16-1 and p16-2R) and the oligonucleotide probe.
g) HPV DNA was examined with consensus primers of the L1 region. HPV types were evaluated by subsequent restriction mapping.
h) HPV-33 DNA was amplified with type-specific primers (p16-1 and p33-2R).[14]
i) Type X; unknown HPV type.
ND; not determined.

of sera from CIN patients (group B), 4% (2/49) of sera from other cancer patients (group C) and 0% (0/41) of sera from non-malignant patients (group D) were positive for antibody against the E7 protein. Among male patients, 0% (0/15) of sera from group C and 10% (1/10) of sera from group D were also antibody-positive. Some other cancer patients belonging to group C and group D showed anti-E7 antibody in their sera. One patient with endometrial cancer, one with breast cancer and one male patient with colon polyp were sero-positive for the anti-E7 antibody (Table I, Fig. 2).

Detection of the HPV DNA in tissues matched to the sera of cervical neoplasia patients Twenty-seven DNA samples in groups A and B were extracted from 15 frozen tissues and 12 paraffin-embedded sections (Table II). PCR techniques were chosen for the detection of the HPV DNA because of the limited quantities of sample DNA (Figs. 3 and 4). We could detect HPV DNA in 26/27 (96%) samples in groups A and B.

We failed to classify HPV from 6 samples into any of the previously reported HPV types by PCR amplification of HPV DNA using consensus primers of the L1 fragment, followed by restriction map analysis of the amplified DNA. We therefore further investigated the HPV types in these samples by partial sequencing.

HPV-16 DNA could be detected in 50% (13/26) of cervical cancer patients and 69.2% (9/13) of these patients had anti-E7 antibody. HPV DNA could be detected in all cases that had anti-E7 antibodies in group A (Table II). Clinical diagnoses of 4 sero-negative cervical cancer patients harboring HPV-16 DNA were stage Ia or Ib, but all patients with stages II, III, and IV harboring HPV-16 DNA had the anti-E7 antibody in their sera. It was also found that HPV-16 DNA could be detected in 90% (9/10) of the cervical cancer patients who had antibody against HPV-16 E7 protein (Table II) and one HPV-16 DNA-negative but sero-positive cervical cancer patient (patient No. 9) harbored HPV-31 DNA. Our purified HPV-16 E7 protein might cross-react with antibody against E7 protein of HPV-31, which is highly homologous to HPV-16 E7. In fact, it was found that HPV-31, -33 and -58 E7 GST fusion protein could cross-react with affinity-purified HPV-16 anti-E7 rabbit serum (data not shown).

Continued presence of anti-E7 antibodies in sera after treatment Three cervical cancer patients, an endometrial cancer patient, and a breast cancer patient who were positive for anti-E7 antibody could be followed up after their treatments (Fig. 2). In all these cases the antibody could always be detected in sera during follow-up for a maximum of more than 2 years (data not shown) after surgical removal of the cancer.

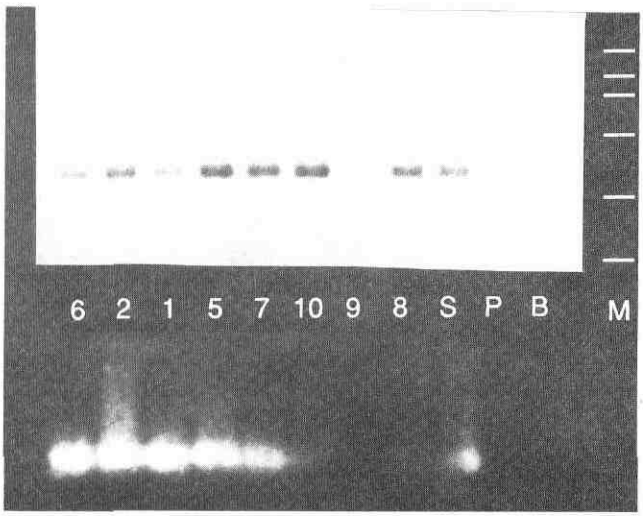

Fig. 3. Amplification of HPV DNA sequences with type-specific primers for the E6 region. Template DNAs (100 ng) were subjected to PCR and one-tenth of the reaction mixture was loaded on 3.5% agarose gel. After electrophoresis, the gels were stained and photographed (upper) and Southern blotted with an internal oligonucleotide probe (lower). The lane numbers correspond to the patients' numbers (Table II). Lane S; template DNA was SKG IIIa (HPV-16 DNA is integrated at 1 copy/cell).[3] Lane P; template DNA was placenta. Lane B; no template DNA. Bars on the right side show molecular markers (ϕX174 digested with *Hae* III): from the top; 310, 281–271, 234, 194, 118, and 72 base pairs.

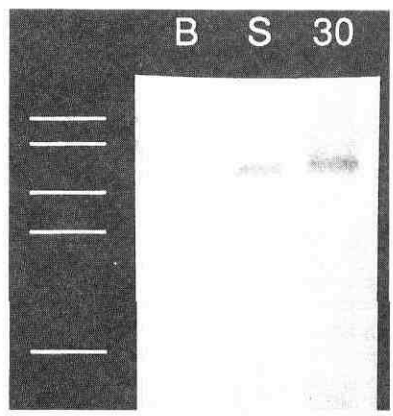

Fig. 4. Amplification of HPV DNA sequences with consensus primers for the L1 region. Template DNAs (100 ng) were subjected to PCR and one-tenth of the reaction mixture was loaded on 3.5% agarose gel. After electrophoresis, the gels were stained and photographed. Lane B; no template DNA. Lane S; template DNA was SKG IIIa (HPV-16 DNA is integrated at 1 copy/cell).[3] Lane 30; paraffin-embedded tissue of the colon polyp patient. Bars on the left side show molecular markers (ϕX174 digested with *Hae* III): from the top; 310, 281–271, 234, 194 and 118 base pairs.

DISCUSSION

Immunological studies on cervical cancer-associated HPV have been hindered by the failure to obtain virion particles from cervical cancer. In addition, HPV cannot be propagated in culture. There have been a few studies on the immune response to HPV infection using fusion protein or synthetic peptides,[17-22] and there are only two reports on measurement of serum antibodies against unfused E7 protein, chiefly using ELISA.[23,24] We have described here the detection of the anti-E7 antibody in sera by Western blot analysis using the unfused, purified, biologically active protein. Synthetic peptides may present smaller antigenic determinants than the unfused purified protein. In addition, the viral epitope might be altered in fusion proteins. Antibodies against HPV-16 early proteins have been reported by using bacterially derived fusion proteins,[21] and it was shown that the occurrence of the anti-E7 IgG was 20.5% in cervical cancer patients, 6.5% in CIN patients and 1.4% in the control group. Recently, antibody against unfused recombinant E7 protein of HPV-16 was reported.[23,24] Ten out of 17 (58%) cervical cancer patients harboring HPV-16 DNA showed anti-E7 IgG antibody in ELISA, and antibody against E7 protein was suggested to be a marker for metastasis. Our data are essentially consistent with those reports. However, it should be noted that the antibody against E7 of HPV-16 could not be found in CIN patients and antibody against E7 protein was not associated with metastasis of cervical cancer (Table II), which is different from previous reports.[21-24]

In our study, all the patients with stages II, III, and IV cervical cancer harboring HPV-16 DNA were seropositive for the anti-E7 antibody, while four out of five cervical cancer patients at stage Ia or Ib were seronegative. These findings suggest that the presence of detectable anti-E7 antibody in serum depends on the staging of cervical cancer. The present results, showing persistence of the anti-E7 antibody for up to 2 years or so after hysterectomy, indicated that the serum antibody against E7, once produced, continued to circulate for long periods of time after removal of the HPV-16-infected lesions, although the possibility that HPV DNA is present in extra-uterine regions can not be ruled out yet. The results presented in this report further indicated that serum anti-E7 antibody detected by the present method could be an indicator of advanced stage, but could not serve as a marker for the relapse of cervical cancer.

Anti-E7 antibody against HPV-16 was found in sera from the colon polyp (patient No. 30). Surprisingly, we could detect the L1 fragment of HPV with L1 consensus primers from this patient's polyps. Sequencing of the L1 fragment showed that it was the same as L1 from HPV-16 and HPV-33. However, we failed to amplify the E6 fragment with type-specific primers for HPV-16 and HPV-33. Other researchers reported the detection of HPV in colon neoplasms by immunohistochemical analysis with anti-bovine papillomavirus type 1 (BPV-1) antibody and *in situ* hybridization with HPV DNA as probes.[25,26] They selected samples reactive with the anti BPV-1 antibody in immunohistochemistry and then performed *in situ* hybridization. They found the HPV genome in 5/18 (27%) adenomas and 9/29 (31%) invasive carcinomas. Further studies will be required to elucidate the involvement of HPV in colon polyp.

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