Human Papillomavirus Type 16 E7 Gene Sequence in Human Cervical Carcinoma Analysed by Polymerase Chain Reaction and Direct Sequencing

Yukako FUJINAGA[1], Kazuhide OKAZAWA[1,3], Yoshitaka OHASHI[1],
Yasushi YAMAKAWA[1,2], Michio FUKUSHIMA[1,2],
Ikunoshin KATO[3], and Kei FUJINAGA[1],

[1] *Department of Molecular Biology, Cancer Research Institute,* [2] *Depart-
ment of Obstetrics and Gynecology, Sapporo Medical College, S1, W17,
Chuo-ku, Sapporo 060 ; and* [3] *Bio Research Laboratories, Takara
Shuzo Co. Ltd., Seta 3-4-1, Otsu, Shiga 520-21*

SUMMARY

Nucleotide sequence diversity of human papillomavirus type 16(HPV16) E7 coding regions present in 7 cervical carcinomas was analysed by direct DNA sequencing of PCR (polymerase chain reaction)-amplified products. Of seven cervical carcinomas, two were completely identical with published prototype HPV16 E7 sequence. Five cases showed sequence diversity from prototype HPV16, and a total of 13 nucleotide exchanges was detected. Four of these led to single amino acid changes (*Thr*-5 to *Lys*-5 in one case and *Asn*-29 to *Ser*-29 in three cases). Our results suggest that a significant level of nucleotide diversity exists in the E7 region.

Key words : Cervical carcinoma, Human papillomavirus, Direct sequencing

INTRODUCTION

Several genital human papillomaviruses (HPVs) are strongly associated with malignant anogenital lesions. In particular, HPV16 is most frequently found in cervical carcinomas(5, 9). A number of reports have now demonstrated that the HPV16 E7 gene encodes the major transforming activity of the virus; expression of E7 gene can transform established rodent fibroblasts(11, 15, 21, 22), immortal-ize primary rat brain cells(12), and cooperate with the activated Ha-ras on-cogene to fully transform primary rat cells(13, 15, 20). It is also apparent that the HPV16 E6 and E7 genes are shown to be necessary and sufficient for transfor-mation of human primary keratinocytes(14). Recent studies have demonstrated that E7 in common with adenovirus E1A and SV40 large T antigen is capable of binding to the 105 K-Da retinoblastoma suppressor gene product (p105-RB)(6).

At the amino acid level E7 shows a similarity with regions of the RB binding domain of adenovirus E1A and SV40LT proteins(6). A recent study using E7 point mutations has identified a region of E7 important for RB binding and shown that mutations defective for RB binding are also unable to transform cells(2). Most recently, other important domains within E7 for transformation activity have been determined by similar point mutation analysis(1, 3, 7, 19, 23). However, there was little information regarding HPV16 E7 gene structure in tumors of the cervix. In this study, we have used an asymmetric PCR (polymerase chain reaction)-direct sequencing method and analysed HPV16 E7 region from 7 cervical carcinomas.

MATERIALS AND METHODS

DNA preparation from clinical samples

Fresh tissue specimens of cervical carcinoma were obtained at the Department of Obstetrics and Gynecology, Sapporo Medical College Hospital between 1985 and 1987, and stored at $-80°C$ before use. Purified DNA was prepared by a standard proteinase K/SDS/ phenol chloroform procedure(8).

Primers

Two 20-base oligonucleotide primers were synthesized on an Applied Biosystems model 380B DNA synthesizer and purified by high-performance liquid chromatography. They are complementary to segments of HPV16 genome and have the following sequences, which are numbered according to Ref.: 17

Sense primer pE7-F   532-5' AAGAACACGTAGAGAAACCC 3'-551
Antisense primer pE7-R   869-5' ATTGGTACCTGCAGGATCAG 3'-888

Amplification of single-stranded DNA

Asymmetric PCR was carried out by the method of Gyllensten and Erlich(10) with minor modifications. Sample DNA (1 μg) was incubated for 10 min at 94°C and chilled quickly on ice for DNA denaturation. Each PCR contained sense primer pE7-F and antisense primer pE7-R (50 pmol of one primer and 0.5 pmol of the other.), 200 pmol each dNTP, 1×PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl$_2$, 100 μg/ml gelatin), and 2.5 units of *Taq* polymerase (Perkin-Elmer Cetus Instruments) in a total volume of 100 μl. Reactions were performed in 0.5-ml microcentrifuge tubes with the Perkin-Elmer Cetus DNA Thermal Cycler. The thermal profile involved 35 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 2 min, and extention at 72°C for 3 min. Amplification was verified by electrophoresis of 1/10 of the reaction volume on a composite gel consisting of 3% NuSieve agarose (FMC Bioproducts) and 1%

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SeaKem agarose (FMC Bioproducts) stained with ethidium bromide.

End-labeling of the primer for the DNA sequencing reaction.

 pE7-F and pE7-R (respectively, 2.5 pmol) were phosphorylated for 30 min at 37°C by T4 polynucleotide kinase (10 units; New England Biolabs) in a total volume of 10 μl of solution containing 50 mM Tris-HCl, pH 7.6, 10 mM MgCl$_2$, 10 mM 2-mercaptoethanol, and 25 μCi of [γ-^{32}P] ATP (3000 Ci/mmol; 10 mCi/ml; New England Nuclear). Reaction mixture was incubated at 94°C for 2 min to inactivate T4 polynucleotide kinase and used for sequencing reaction without purification.

Sequencing of PCR products.

 Prior to sequencing, PCR-amplified DNA samples were purified as follows. PCR reaction mixtures were extracted once with phenol/chloroform and ethanol precipitated. Dried pellets were dissolved in 100 μl of TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and mixed with 0.6 volumes of PEG-NaCl (20% w/v polyethylene glycol 6000, 2.5 M NaCl), and incubated for 1 hr at 0°C. Nucleic acid precipitates were collected by centrifugation at 12000 g for 5 min at 4°C. These pellets were washed twice with 80% ethanol, then dried in a Speed Vac Concentration (Savent) and resuspended in 10 μl of TE. Two μl of them were analyzed by electrophoresis on a composite gel consisting of 3% NuSieve agarose (FMC Bioproducts) and 1% SeaKem agarose (FMC Bioproducts) stained with ethidium bromide. Then, about 8 μl of remained samples were directly sequenced by a modified method of Sanger et al.(16) using a "Sequenase" Kit (United States Biochemical). Two μl of ^{32}P-labeled pE7-F or pE7-R (0.5 pmol) was annealed to the amplified DNA in a total volume of 12 μl containing 2 μl of 5× sequenase buffer by heat denaturing the strands at 60°C for 15 min and gradual cooling to 37°C. One μl of 0.1 M dithiothreitol, One μl of H$_2$O and 2 μl of sequenase (3 units) were then added to the template-primer mixture. 3.5 μl of this mixture was divided into four tubes containing 2.5 μl of dideoxy A, G, C, and T termination mixtures and the reaction was allowed to proceed for 20 min at 37°C. Four μl of stop solution was added to each tube and mixture was heat-denatured at 94°C for 3 min and immediately cooled on ice. Three μl of the mixtures were subjected to electrophoresis on 8 M urea/6% acrylamide gels at 40 watts for 1.5-6.5 h. Gels were dried and exposed to X-ray film (Kodak, X-OMAT AR) at-80°C for 12-24 h without screen intensification.

RESULTS and DISCUSSION

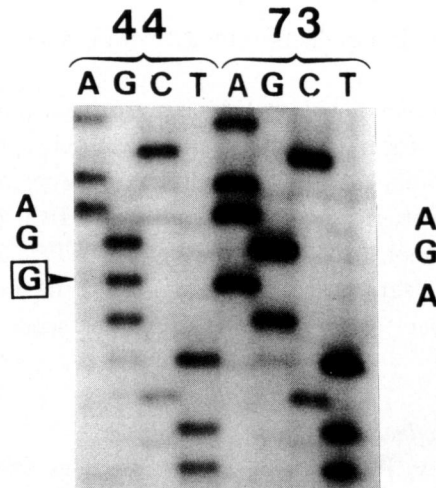 HPV16 DNAs from seven cervical carcinomas were amplified by asymmetric

Fig. 1 Sequencing autoradiogram of antisense strand of PCR-amplified E7 gene in carcinoma No. 44 and No. 73. E7 from carcimona No. 44 contains the sequence GGA at codon 95, which represents a T to C transition at the third position in the coding strand.

PCR with pE7-F/R primer pair and directly sequenced along sense and antisense strand of E7 coding region completely from ATG initiation codon to TAA termination codon. This region from each carcinoma, 297 nucleotides in length was compared with prototype HPV16 sequenced by Seedorf et al.(17). Representative results from the sequencing autoradiogram are shown in Fig. 1 In the case of NO. 44, T to C exchange at HPV16 nucleotide 846 was observed. A total of 7 cervical carcinomas was then analysed. Of 7 cases, two were completely in agreement with the nucleotide sequence of prototype HPV16 DNA. Five cases showed sequence diversity from prototype HPV16, and a total of 13 nucleotide exchanges was observed (Table 1). Four of these would result in a change of the encoded amino acid residues (*Thr*-5 to *Lys*-5 and *Asn*-29 to *Ser*-29). Our analysis reveals that a significant level of nucleotide diversity exists in the E7 region. In particular, A to G transition at nucleotide 647 and T to C transition at nucleotide 846 were discovered in 3 and 4 cases, respectively. In agreement with these results, same exchanges have also been observed at these sites (nucleotide position 647 and 846) in a cervical cell line QG-U by Shirasawa et al. (18), and at nucleotide position 846 in a cervical carcinoma obtained from a Chinese national patient (surgical sample) by Choo et al.(4). It is interesting to determine whether the amino acid exchanges at *Thr*-5 and *Asn*-29 could alter the transforming activity of E7 protein. As shown in Fig. 2, *Thr*-5 and *Asn*-29

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Table 1 *Sequence diversity of HPV16 E7 gene in human cervical carcinomas*

Patient No.	Position	Codon	Resulting amino acid exchange
2	575	ACA* → AAA	Thr[5]* → Lys[5]
	732	TTT → TTC	Phe[57] —
	789	ATT → ATC	Ile[76] —
	795	ACT → ACG	Thr[78] —
44	647	AAT → AGT	Asn[29] → Ser[29]
	828	ATT → ATC	Ile[89] —
	846	TCT → TCC	Ser[95] —
76	647	AAT → AGT	Asn[29] → Ser[29]
	846	TCT → TCC	Ser[95] —
80	647	AAT → AGT	Asn[29] → Ser[29]
	846	TCT → TCC	Ser[95] —
179	663	GAG → GAA	Glu[34] —
	846	TCT → TCC	Ser[95] —

* ; Prototype HPV16
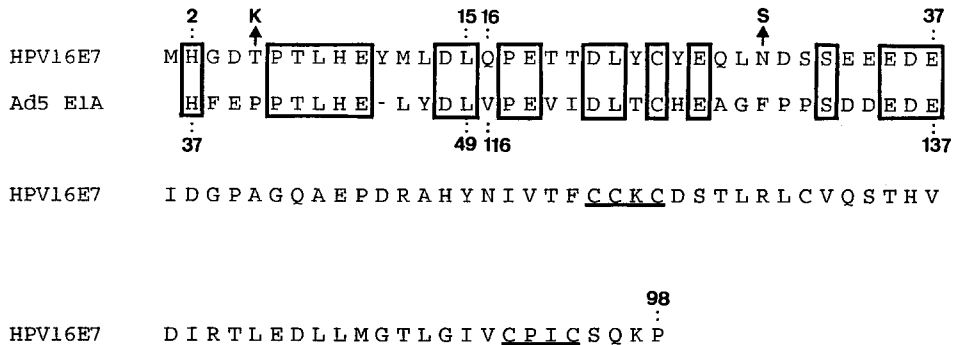
Fig. 2 Amino acid sequence of HPV16 E7. Identical amino acids with adenovirus E1A (Ad E1A) are boxed according to Phelps et al.(15). -Cys-X-X-Cys- motifs are underlined. Amino acid exchanges detected in this study are shown above the corresponding E7 residues.

are not in the regions homologous to E1A(15) nor-Cys-X-X-Cys-core of a 'zinc finger' motifs. So it seems these exchanges would not affect transforming activity of E7 protein. Recent studies using E7 point mutations have identified regions of E7 important for its transforming activity (1, 2, 3, 7, 19, 23). However, the mutations at *Thr*-5 and *Asn*-29 have not been investigated. Clearly, it is now important to test the transforming activity of the E7 variant proteins ([Lys[5]] E7

protein and [Ser^{29}] E7 protein) by *in vitro* assays, such as co-operation with ras to transform primary rodent cells.

REFERENCE

1. BANKS, L., EDMONDS, C. and VOUSDEN, K. H.: **Oncogene 5**, 1383-1389 (1990).
2. Barbosa, M. S., EDMONDS, C., FISHER, C., SCHILLER, J. T., LOWY, D. R. and VOUSDEN, K. H.: **EMBO J. 9**, 153-160 (1990).
3. CHESTERS, P. M., VOUSDEN, K. H., EDMONDS, C. and MCCANCE, D. J.: **J. Gen. Virol. 71**, 449-453 (1990).
4. CHOO, K. B., LEE, H. H., PAN, C. C., WU, S. M., LIEW, L. N., CHEUNG, W. F. and HAN, S. H.: **J. Virol. 62**, 1659-1666 (1988).
5. DÜRST, M., GISSMANN, L., IKENBERG, H. and zur HAUSEN, H.: **Proc. Natl. Acad. Sci. USA 80**, 3812-3815 (1983).
6. DYSON, N., HOWLEY, P. M., MÜNGER, K. and HARLOW, E.: **Science 243**, 934-937 (1989).
7. EDMONDS, C. and VOUSDEN, K. H.: **J. Virol. 63**, 2650-2656 (1989).
8. FUKUSHIMA, M., YAMAKAWA, Y., SHIMANO, S., HASHIMOTO, M., SAWADA, Y. and FUJINAGA, K.: **Cancer 66**, 2155-2161 (1990).
9. GISSMANN, L., WOLNIK, L., IKENBERG, H., KOLDOVSKY, U., SCHNÜRCH, H. G. and zur Hauzsen, H.: **Proc. Natl. Acad. Sci. USA 80**, 560-563 (1983).
10. GYLLENSTEN, U. B. and ERLICH, H. A.: **Proc. Natl. Acad. Sci. USA 85**, 7652-7656 (1988).
11. KANDA, T., FURUNO, A. and YOSHIIKE, K.: **J. Virol. 62**, 610-613 (1988).
12. KANDA, T., WATANABE, S. and YOSHIIKE, K.: **Virology 165**, 321-325 (1988).
13. MATLASHEWSKI, G., SCHNEIDER, J., BANKS, L., JONES, N., MURRAY, A. and CRAWFORD, L.: **EMBO J. 6**, 1741-1746 (1987).
14. MÜNGER, K., PHELPS, W., BUBB, V., HOWLEY, P. M. and SCHLEGEL, R.: **J. Virol. 63**, 4417-4421 (1989).
15. PHELPS, W. C., YEE, C. L., MÜNGER, K. and HOWLEY, P. M.: **Cell 53**, 539-547 (1988).
16. SANGER, F., NICKLEN, S. and COULSON, A. R.: **Proc. Natl. Acad. Sci. USA 74**, 5463-5467 (1977).
17. SEEDORF, K., KRÄMMER, G., DÜRST, M., SUHAI, S. and RÖWEKAMP, W. G.: **Virology 145**, 181-185 (1985).
18. SHIRASAWA, H., TOMITA, Y., FUSE, A., YAMAMOTO, T., TANZAWA, H., SEKIYA, S., TAKAMIZAWA, H. and SIMIZU, B.: **J. Gen. Virol. 79**, 1913-1919 (1989).
19. STOREY, A., ALMOND, N., OSBORN, K. and CRAWFORD, L.: **J. Gen. Virol. 71**, 965-970 (1990).
20. STOREY, A., PIM, D., MURRAY, A., OSBORN, K., BANKS, L. and CROWFORD, L.: **EMBO**

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J. **7**, 1815–1820 (1988).

21. TANAKA, A., NODA, T., YAJIMA, H., HATANAKA, M. and ITO, Y.: **J. Virol. 63**, 1465–1469 (1989).

22. VOUSDEN, K. H., DONIGER, J., DIPAOLO, J. A. and LOWY, D. R.: **Oncogene Res. 3**, 167–175 (1988).

23. WATANABE, S., KANDA, T., SATO, H., FURUNO, A. and YOSHIIKE, K.: **J. Virol. 64**, 207–214 (1990).