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Sequence variants and functional analysis of human papillomavirus type 16 E5 gene in clinical specimens

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Summary. Previously, we found that the E5 protein can be expressed in HPV-16 infected precancerous lesions and cervical cancer [4]. In this study, we investigated the presence of sequence variants of E5 in HPV-16 infected tissues. Toward this end, we amplified the E5 gene by polymerase chain reaction from 29 HPV-16 infected tissues including eight normal tissues, seven high grade neoplastic tissues (high grade squamous intraepithelial lesions (HSIL) and 14 cervical cancer tissues. Sequence analysis demonstrated that there were three mutational hot spots at positions 3979, 4042, and 4077 of the HPV-16 DNA; these and other mutations resulted in six variants in the E5 sequence. This resulted in four E5 protein mutants, named WTE5 [wild type E5 protein], 14E5, 21E5 and 56E5. Functional analysis of these four mutant proteins revealed that the transforming activities of 14E5, 21E5 and 56E5 were 0.95, 0.59, and 0.89 fold of WTE5, respectively. Although E5 was expressed in all of the HSIL and cervical cancer tissues, but in only one of the eight normal tissues tested, only WT E5 protein was found in HSIL while in cervical cancer tissues both WT and mutant E5 proteins were detected. Since these E5 proteins exhibited the same in vitro transforming activity, these data suggest that expression of E5 is important in development and progression toward malignancy but mutation of E5 does not affect the transformation process.

Introduction

HPV-16 is the etiologic agent of cervical cancer and encodes three transforming oncogenes-E5, E6, and E7 [19, 32]. During viral infection, E5 is expressed at earlier stages of neoplastic transformation of the cervical epithelium than are E6 and E7 [29]. E5 is a highly hydrophobic protein and is found in the membrane compartment of cells [13, 24]. Recent reports have shown that the E5 protein of HPV-16 can increase the half life of the EGF receptor [30]. The tyrosine kinase growth factor receptors, such as the EGF receptor, initiate diverse biochemical events that ultimately result in the transcription of a variety of proto-oncogenes. Previously, it was shown that the expression of c-jun, junB and c-fos is induced by the E5 protein [1, 6–8, 10], suggesting E5 potentiation of signaling pathways downstream from growth factor receptors [14, 20, 22, 28]. Moreover, the HPV-16 E5 gene in human keratinocytes has been shown to reduce cell-cell gap junctional communication, indicating that E5 may render cells insensitive to normal growth regulation signals [27].

The E5 gene has been reported to exhibit great diversity among HPV-6 subtypes [26]. To determine whether HPV-16 infected tissues contain divergent E5 sequences, we investigated the sequence variation of E5 from HPV-16 infected tissues which we had previously characterized [5]. To this end, we amplified the E5 region by polymerase chain reaction (PCR) from 29 HPV-16 infected tissues. The result of DNA and amino acid analysis identified four HPV-16 E5 protein mutants. We examined the biological activity of these four E5 mutants by anchorage-independent growth in cells transfected with these mutants. In addition, we correlated the transforming activity of the E5 protein with different clinical stages of cervical cancer.

Materials and methods

Specimens, PCR and cloning

Previously, we had identified HPV types by PCR and restriction fragment length polymorphism [5]. In this study, we examined whether HPV-16 infected tissues exhibited E5 sequence divergence by amplifying the sequence from 29 HPV-16-infected frozen tissues including eight normal tissues, seven high grade squamous intraepithelial lesions (HSIL) and 14 cervical cancers [3, 4]. These normal cervical tissues were collected from patients who underwent hysterectomies for reasons other than malignant cervical disease. One μg of DNA purified from each of the above biopsy specimens was amplified by PCR as described previously [9] by high fidelity Taq DNA polymerase (Promega) using a forward 30 mer primer (GAACTTGGATCCTACTGGATTTATGTCTAT) containing a BamHI sequence (underlined) and the 18 nucleotides preceding the E5 initiation codon and a reverse 30 mer primer (TTCGAGGGATCCACATTATGTACATATACA) containing a BamHI site (underlined) and 18 nucleotides downstream from the E5 stop codon. The PCR amplification products were introduced into a pGemT vector (Promega) and sequenced in both directions by the dideoxynucleotide method using T7 and SP6 primers (Promega).

Construction of HA1 epitope tag-E5 mutant plasmids in the eukaryotic expression vector pCEP4

Each E5 mutant was reamplified by PCR from the appropriate pGemT templates using as an upstream primer GAACTTGGATCCATGTACCCATA CGATGTTCCAGATTACGCTAGCTTGATGACAAATCTTGAT and as a downstream primer TTCGAGGGATCCTTATGTAATTAAAAAGCG and cloned into pCEP4 vector (Invitrogen). The upstream primer contains the BamHI restriction site, the sequences for the HA1 epitope, and the first six codons of the E5 open reading frame (ORF); The downstream primer contains the BamHI sequence and 18 nucleotides which are complementary to the last six codons of E5 ORF. The resulting CHA16E5, 14E5/pCEP4. 21E5/pCEP4 and 56E5/pCEP4 plasmids contain the WTE5, 14E5, 21E5 and 56E5 sequences (Fig. 1), respectively, with the nucleotide sequence encoding the HA1 epitope in frame at the 5′ end of each E5 sequence. The HA1 epitope tag is an 11 amino-acid sequence [Met-Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala-Ser] from a hemagglutinin epitope of influenza virus, against which a highly reactive monoclonal antibody is available (ATCC). These plasmids all carry a hygromycin resistant marker and utilize the human cytomegalovirus immediate early gene promoter to direct the gene expression.

Cell culture and transfection

CX cells, established from a patient with squamous cell carcinoma of the uterine cervix, were HPV-negative, unable to grow in soft agar and non-tumorigenic in nude mice [12]. CX cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics. Transfections by calcium phosphate precipitation were carried out as previously described [7]. Hygromycin resistant colonies (at least 80 colonies) were pooled and expanded. Cell morphology was observed under phase contrast microscope.

Analysis of E5 protein expression

Cell extracts were prepared as described previously [9]. The antibody to the HA1 epitope (ATCC, 12CA5 culture medium 100 μg/ml) was added to extracts, which were then incubated for 2 h at 4°C with rotation. 50 μl of a 1:1 suspension of protein A-Sepharose beads (Pharmacia) in TBS-BSA [10 mM Tris-HCl, pH 7.4, 165 mM NaCl, 10% (wt/vol) bovine serum albumin] was added and the mixture was rotated for 45 min at 4 °C. The beads were pelleted and washed five times with cold RIPA buffer [50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS)] plus protease inhibitors [phenylmethyl sulfonyl fluoride (PMSF), leupeptin], and resuspended in 75 μl of sample buffer with β-mercaptoethanol. Samples were heated to 100 °C for 4 min and analyzed by 15% SDS-PAGE. Gels were subjected to immunoblot analysis with HA1 mouse monoclonal antibodies (1:500 dilution, 100 μg/ml) and visualized by an enhanced chemoluminacent system (Amersham) using procedures recommended by manufacturer.

Immunohistochemistry assays

Avidin-biotin immunohistochemistry was performed on 4 μm sections from routinely processed paraffin-embedded tissues as described previously [3]. The E5 antibody we generated previously against the combination of two synthetic peptides which cover the entire E5 protein is specific for the HPV-16 E5 protein [4]. The sections were incubated with E5 antibody (1:50 dilution, 100 μg/ml) for 1 h at room temperature, then rinsed with PBS, incubated with biotinylated anti-mouse antibody (1: 100 dilution; DAKO) for 1 h at room temperature, rinsed with PBS and incubated with avidin-biotin complex (DAKO) for 30 min at room temperature. The substrate-chromogen, 3% amino-9-ethylcarbazone (DAKO) was developed

for 3 to 5 min. The slides were then rinsed in running tap water, counterstained with Mayer hematoxylin, dehydrated, and coverslipped.

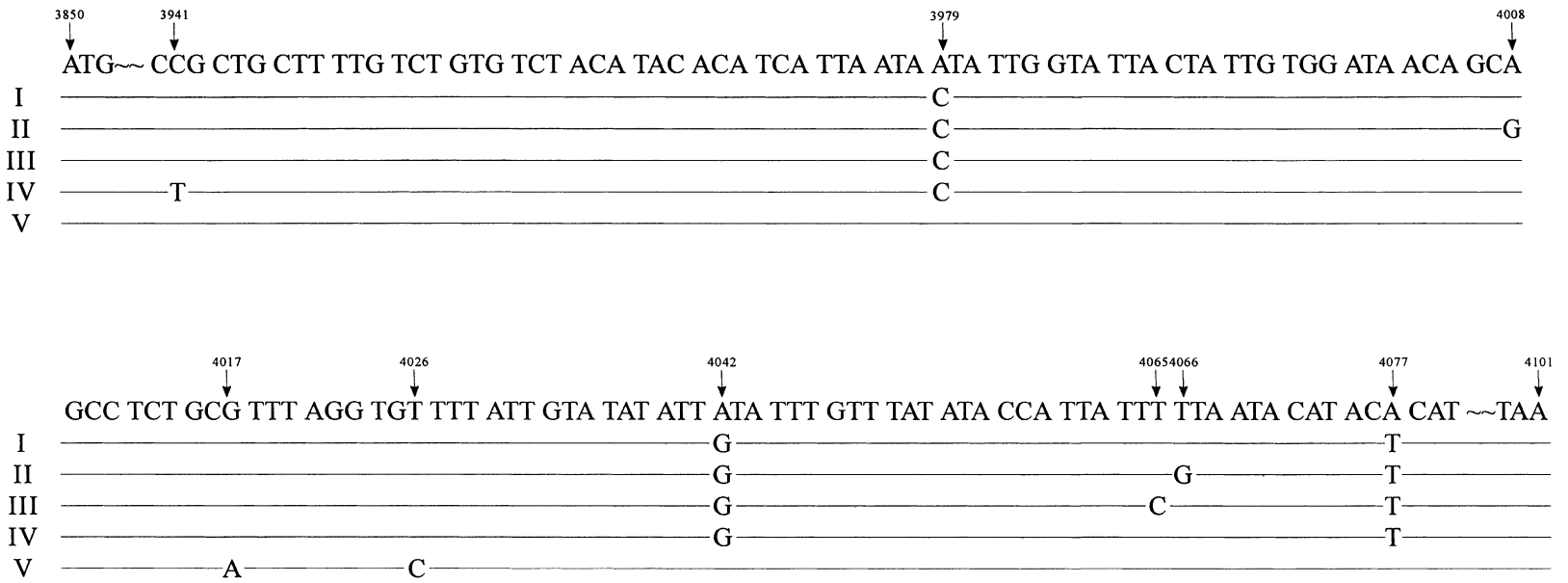
Results

HPV-16 E5 sequence variants

To determine whether HPV-16 infected tissues contain divergent sequences in the E5 gene, we amplified the E5 gene from 29 HPV-16-infected tissue samples by PCR and then sequenced by the dideoxynucleotide method between positions 3850 to 4101 of the HPV genome, a region encompassing the complete E5 ORF. To exclude potential mutations as a result of PCR, we amplified HPV-16 the E5 DNA fragment from plasmid DNA in several independent experiments and determined the nucleotide sequence. As each test result was identical, i.e., the sequence was wt, we concluded that the observed mutations were originally present in the intrachromosomal HPV DNA copies and represented the natural diversity of HPV-16 genomes. In comparison with the published sequence [16], E5 from the 29 HPV-16-infected tissues showed only base changes but no deletions or insertions. These were grouped as shown in Fig. 1; type 1 sequence included 3 base substitutions (1.2% sequence divergence) (six cases: C14, C18, C23, C32, C39, and N262); the type II sequence contained 5 base changes (2% sequence divergence) (one case: C21); type III had 4 base changes (1.6% sequence divergence) (one case: C35); type IV included 4 base changes (1.6% sequence divergence) (one case: C56); type V included 2 base changes (0.8% sequence divergence) (14 cases); and the remaining six cases had the wild type E5 sequences. In sum, we found six different variants of the E5 sequence.

E5 amino acid sequence mutants and correlation with disease distribution

Aligning the amino acid sequence of these six E5 DNA variants revealed only four mutant protein sequences, named WTE5, 14E5, 21E5 and 56E5. As shown in Table 1, WTE5 included the WT, type III and Type V DNA sequence variants which encoded the wild type E5 protein; 14E5 was encoded by the type I DNA sequence variant and contained aa (amino acid) mutations at position 44 I to L and 65 I to V; 21E5 was encoded by the type II sequence variant and had aa mutations at position 44 I to L, 65 I to V, and 73 L to V; and 56E5 was the type IV DNA sequence variant in which E5 was mutated at position 31 P to L, 44 I to L and 65 I to V. As shown in Table 2, HPV in 21 out of 29 tissues contained the wild type E5 protein sequence; including seven normal tissues, seven high grade squamous intraepithelial lesions and seven cervical cancer tissues. Five cases of cervical cancer tissues had HPV with mutant 14E5 protein sequence. The 21E5 and the 56E5 mutant protein sequence each occurred in one case of cervical cancer. Thus E5 protein mutants were detected more frequently in the cancer (6/14) than in the normal tissues (1/8) and HSIL (0/7), however this difference is not significant by Fisher's exact test. Since the sample size is small, in the future, more samples are necessary to determine statistical significance.



I: C14*, C18, C23, C32, C39, N262
II: C21
III: C35
IV: C56
V: C31, C37, C40, C53, C54, D19, D41, D43, D56, N103, N123, N145, N174, N273
WT:C19, D28, D74, D102, N116, N129

Fig. 1. HPV-16 E5 DNA sequence variants from clinical specimens. DNA extracted from 29 HPV-16 infected tissues was used as a template for amplification by PCR. The sequence from positions 3850 to 4101 of the HPV-16 genome encompassing the complete E5 ORF was determined. Six E5 DNA variants were identified. The base changes in these E5 DNA variants are shown below the published sequence of HPV-16 E5 (EMBL accession number K02718; [16]). * presents case number. *N* Normal case; *D* HSIL; *C* cervical cancer

Table 1. HPV-16E5 mutant proteins

aa location				
E5 protein mutants	31	44	65	73
WTE5	P	I	I	L
14E5	–	L	V	–
21E5	–	L	V	V
56E5	L	L	V	–

The amino acid (aa) sequence of these E5 DNA variants was aligned with the published sequence of HPV-16 E5 DNA. Accordingly, there are four different E5 mutant proteins including wild type E5 protein (WTE5)

Table 2. The disease distribution of these E5 mutant proteins

Disease				
E5 mutants	Normal	HSIL	CC	Total
WTE5	7	7	7	21
14E5	1	–	5	6
21E5	–	–	1	1
56E5	–	–	1	1
Total	8	7	14	29

The disease distribution of E5 protein mutants was according to which clinical specimen the DNA was extracted from

HSIL High grade squamous intraepithelial lesion

CC Cervical carinoma

Plasmid construction and soft-agarose assay

To determine if these mutations would affect E5 protein function, we performed a growth in soft-agarose assay to evaluate transforming activity. We added a HA1 epitope at the amino end of these E5 mutants and cloned each HA1 tagged E5 DNA into the eukaryotic expression vector, pCEP4. The constructs were named CHA16E5, 14E5/pCEP4, 21E5/pCEP4 and 56E5/pCEP4, respectively.

To establish cells containing each mutant E5 DNA, CX cells were transfected separately with the E5 plasmids or vector pCEP4 DNA followed by hygromycin selection. After 2–3 weeks at least 80 hygromycin resistant colonies from each transfection were pooled and called CX-WTE5, CX-14E5, CX-21E5, CX-56E5 and CX-V cells, respectively. These were assayed for anchorage-independent growth in soft agar. As shown in Table 3, 34.5, 142, 134.5, 83.5, and 126 colonies per 8000 cells were formed by the CX-V, CX-WTE5, CX-14E5, CX-21E5, CX-56E5 cells respectively. To comparison with wild type E5, 14E5, 21E5 and 56E5

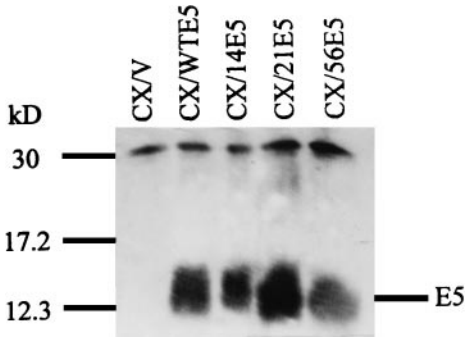


Fig. 2. E5 and mutant E5 protein expression in transfected cells Extracts (100 μg) of CXs cells transfected with vector (CX/V) or vector expressing wt or mutant E5 protein were immunoprecipitated with HA1 antibody, separated electrophoretically by 15% SDS-PAGE, immunoblotted, and detected with HA1 antibody

showed 0.95, 0.59 and 0.89 of the transforming activity in two different experiments. By ANOVA test, the transforming activity of 14E5 and 56E5 was not significantly different from WTE5, but that of 21E5 was significantly different (lower) from WTE5.

E5 protein expression

To exclude the possibility that the decreased transforming activity of the 21E5 mutant was due to lower expression of this mutated gene in transfected cells, we measured the amount of E5 protein in cells containing different mutant by immunoprecipitation/immunoblot assay. As shown in Fig. 2, E5 protein was expressed at higher levels in CX/21E5 cells than in the other E5 containing cells. However, anchorage-independent growth was least in the CX/21E5 cells (Table 3). This suggests 21E5 is actually less active in transformation. The other E5 mutant-containing cells produced similar amounts of E5 protein.

Correlation between E5 protein expression and clinical disease

Previously, we examined E5 protein expression in HPV-16-infected tissues which contained the episomal HPV-16 DNA by immunohistochemistry assay [4]. In this study, we correlated the E5 protein expression with different clinical stages of

Table 3. Anchorage independent growth

E5 mutants					
Experiments	V	WTE5	14E5	21E5	56E5
1	46	142	113	93	150
2	23	142	146	74	102
Mean (±SD)	34.5(±17.95)	142(±20.75)	129.5(±27.14)	83.5(±15.76)	126(±31.16)

Numbers given are averages of triplicate determinations from each experiment. Triplicate dishes were seeded with 8×10^3 cells, adn after three weeks, colonies in soft agarose were counted by more than 16 cell per colony

SD Standard deviation

Table 4. Correlation between E5 protein expression and clinical disease

E5 protein mutants	Case no.	Disease	E5 protein expression
	C14	CC[a]	+
	C18	CC	+
14E5	C23	CC	+
	C32	CC	+
	C39	CC	+
	N262	Normal	−
21E5	C21	CC	±
56E5	C56	CC	+
	C19	CC	+
	C31	CC	+
	C35	CC	+
	C37	CC	+
	C40	CC	+
	C53	CC	+
	C54	CC	+
	D19	HSIL[b]	+
	D28	HSIL	+
	D41	HSIL	+
WTE5	D43	HSIL	+
	D56	HSIL	+
	D74	HSIL	+
	D107	HSIL	+
	D102	HSIL	+
	N103	Normal	−
	N116	Normal	−
	N123	Normal	+
	N129	Normal	−
	N145	Normal	−
	N174	Normal	−
	N273	Normal	−

[a]CC represents cervical carcinoma
[b]HSIL represents high grade squamous intraepithelial lesion

cervical cancer. Table 4 shows that in the 14 cases of HPV-16-infected cervical carcinoma, E5 protein was expressed; however, in the one case with the 21E5 mutant only a small amount of E5 protein could be detected. All seven cases of high grade neoplastic tissues exhibited E5 protein expression, but in only one of the eight normal cervical tissues could the expression of E5 protein be detected.

Discussion

In this study, we sequenced E5 DNAs from 29 HPV-16-infected tissues. Compared with the published sequences of HPV-16 E5 (252 nucleotides), we found only base changes but no deletion/insertions. There were six types of DNA sequence

variants (Fig. 1) which resulted in four mutant E5 proteins (Table 1). We also found that mutations at position 3979, 4042, and 4077 of HPV-16 E5 DNA occurred multiple times. Several combinations of these mutations were found more than once (Fig. 1). These three mutational hot spots had been reported in HPV-16 isolates from Singapore, Brazil, Tanzania, Germany and the United States [2, 17]. If mutational events occurred randomly in a particular patient or a particular population, we would expect a random distribution of mutations throughout the analyzed region [18]. Taken together, it appears that the E5 DNA sequence contains mutational hot spots, which may increase the likelihood of finding specific changes. However, we cannot rule out that mutations in particular positions may arise rarely or only once over long periods of time. Then that particular variant may subsequently spread in a population, itself becoming the target of further mutations that affect different nucleotides.

Since HPV DNA has usually been identified in integrated form in biopsy specimens of cervical cancers and in cervical carcinoma cell lines and E5 is generally not expressed from integrated HPV DNA, this has been taken as evidence that E5 is not essential for cervical cancer [15, 16, 23]. However, unlike other high risk HPVs, recent reports have identified that HPV-16 DNA is present in episomal, integrated, and episomal/integrated forms in primary cervical cancers [11, 15, 25]. In this study, we correlated the E5 protein expression in tissues which had episomal HPV-16 DNA [4] with their clinical disease distributions. Among 14 cases of HPV-16-infected cervical cancer, seven cases expressed the wild type E5 protein, five cases had the 14E5 mutant protein, one case each of the 21E5 and 56E5 mutant protein (Table 2). The transforming activities of the 14E5 and 56E5 mutants are almost the same as the wild type E5; only the transforming activity of 21E5 is lower (about 64% of wild type E5) (Table 3). E5 transformation is reported to be associated with the extending the half life of EGF receptor [30] and are currently investigating this correlation by these E5 mutants.

In addition, all of these 14 cancerous specimens expressed E5 protein as detected by immunohistochemistry assay (Table 4), except in case No 21 which only expressed a slight amount of E5 protein. However, we cannot exclude the possibility that there may be the conformational change of the 21E5 mutant which causes the E5 antibody not to recognize it. In summary, 13 out of 14 cases of HPV-16-infected cervical cancers express the E5 protein, the transforming activity of which are almost the same as the wild type E5 protein. However, in our previous report we demonstrated that 40% of cervical cancers which contain the integrated form of HPV-16 DNA cannot express the E5 protein [4]. Hence, the precise role of the E5 protein in HPV-16 infected cancer is worthy of further investigation.

From previous reports, 80–90% of precancerous lesions express the E5 protein, suggesting that E5 plays an important role in the initial stage of cervical cancer [4, 21]. In this study, seven cases of HSIL expressed E5 protein (Table 4). This finding provides evidence for the transforming role of the E5 in precancer lesions. In addition, in eight normal cervical tissues we could find the expression

of the E5 protein in only one case but not in other seven cases; also suggesting that E5 expression is important in the disease process.

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