筑　波　大　学

博　士　（医　学）　学　位　論　文

Separate analysis of human papillomavirus E6/E7 transcript variants in liquid-based cytology samples from patients with cervical neoplastic diseases

（子宮頸部腫瘍性疾患患者からの液状細胞診検体における
ヒトパピローマウイルスE6/E7転写変異体の個別解析）

２０１８

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List of Abbreviations

B2M	β2-microglobulin
cDNA	Complementary deoxyribonucleic acid
CIN	Cervical intraepithelial neoplasia
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
HPV	Human papillomavirus
HR-HPV	High risk- human papillomavirus
HC2	Hybrid Capture 2
LBC	Liquid-based cytology
mRNA	Messenger ribonucleic acid
NC	Negative control
NPV	Negative predictive value
PCR	Polymerase chain reaction
PPV	Positive predictive value
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
RFLP	Restriction fragment length polymorphism

ABSTRACT

[Objective] A few studies previously suggested that human papillomavirus (HPV) E6 messenger RNA (mRNA) may exist almost uniformly in all grades of cervical intraepithelial neoplasia (CIN), whereas the detection rate of E7 mRNA may increase with disease progression from low-grade CIN to invasive carcinoma. The aim of this study was to clarify the different roles of E6 and E7 mRNAs in the pathogenesis for cervical cancer.

[Methods] The presence of each E6 and E7 mRNA was analyzed in liquid-based cytology samples from 171 patients with pathologically-diagnosed CIN or cervical carcinoma. We utilized a RT-PCR assay based on consensus primers which could detect E6 mRNA (full-length E6/E7 transcript) and E7 mRNAs (spliced E6*I/E7 and E6*II/E7 transcripts) separately for various HPV types.

[Results] E7 mRNAs were detected in 6% of CIN1, 12% of CIN2, 24% of CIN3, and 54% of cervical carcinoma. Presence of E7 mRNAs was significantly associated with progression from low-grade CIN to invasive carcinoma in contrast with present E6 mRNA or positive high-risk HPV (HR-HPV) DNA showing no such trends ($p=0.00011$, 0.80 and 0.54, respectively). Presence of both E6 and E7 mRNAs was significantly associated with positive HPV16/18 DNA but not with positive HR-HPV DNA ($p=0.0079$ and 0.21, respectively), while presence of E6 mRNA was significantly associated with positive HR-HPV DNA but not with positive HPV16/18 DNA ($p=0.0036$ and 0.089, respectively). Presence of both E6 and E7 mRNAs

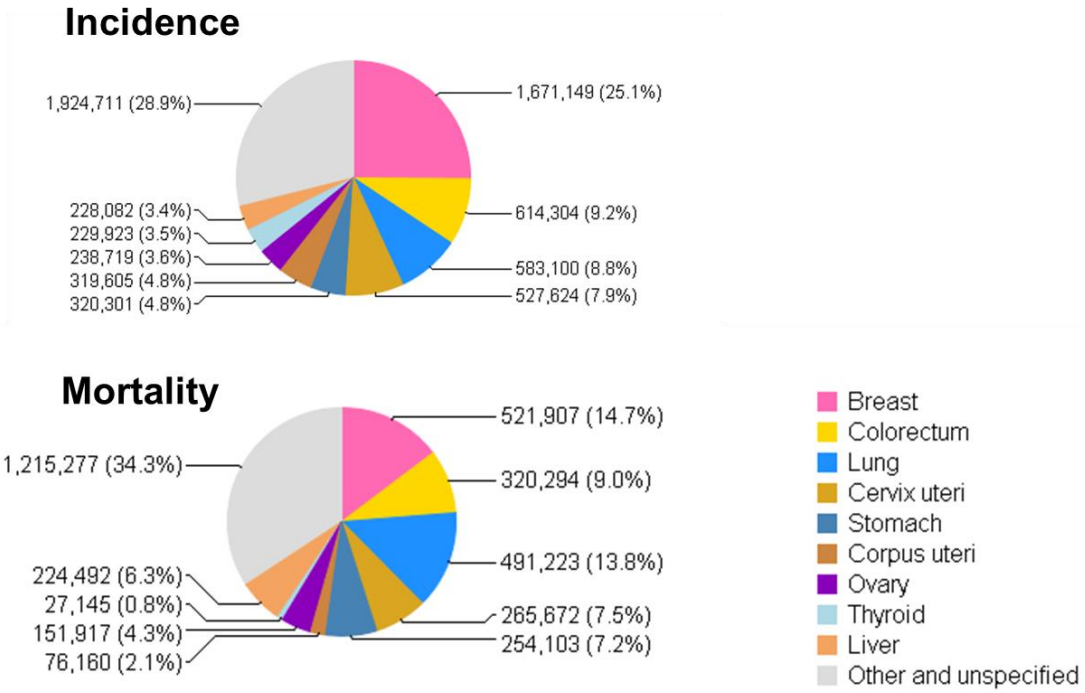
showed high specificity and low sensitivity (100% and 19%) for detecting CIN2+ by contrast with positive HR-HPV DNA showing low specificity and high sensitivity (19% and 89%). The positive predictive value for detecting CIN2+ was even higher by the presence of both E6 and E7 mRNAs than by the positivity for HR-HPV DNA (100% vs. 91%). Furthermore, in 31 patients followed up for CIN1-2, presence of both E6 and E7 mRNAs showed significant association with the occurrence of upgraded abnormal cytology in contrast with E6 mRNA, HR-HPV DNA, or HPV16/18 DNA showing no such trends (p=0.034, 0.73, 0.53, and 0.72, respectively).

[Conclusion] Our findings support previous studies according to which E7 mRNA is more closely involved in cervical carcinogenesis than E6 mRNA, and the presence of both E6 and E7 mRNAs may exert the strongest transforming ability. Moreover, the separate analysis of E6 and E7 mRNAs may be more useful than HR-HPV DNA test for detecting CIN2+ precisely and predicting disease progression. Further accumulation of evidence is warranted to validate our findings.

1. INTRODUCTION

Cervical cancer is the fourth most commonly diagnosed cancer and the fourth most common cause of cancer death in women worldwide (Fig. 1) [1]. Each year, 528,000 women develop cervical cancer, and 266,000 women die of the disease, accounting for 7.5% of all cancer deaths in females (Fig. 1) [1].

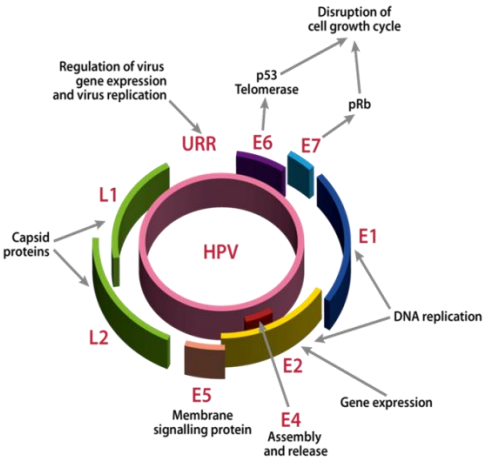
Fig. 1. The incidence and mortality of cervical cancer in women worldwide. (http://gco.iarc.fr/today/home)



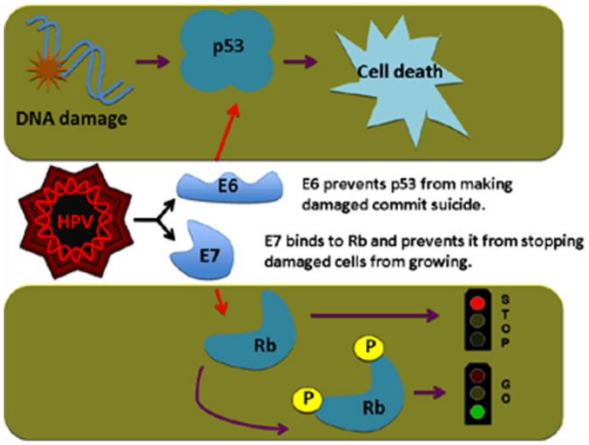
GLOBOCAN 2012 (IARC) Section of Cancer Surveillance (10/5/2015)

Human papillomavirus (HPV) is classified by the sequence of the L1 gene. Infection with high-risk HPV (HR-HPV), including types 16 and 18, causes development of low-grade cervical intraepithelial neoplasia (CIN), and viral persistence induces cellular transformation resulting in progression to high-grade CIN and invasive cervical cancer [2]. HPV viral genome has 6 early genes, E1, E2, E4, E5, E6, and E7, and 2 late genes, L1 and L2, encoding capsid proteins (Fig. 2). Among the early genes, E6 and E7 cause cancer by inactivating the tumor suppressor proteins p53 and Rb via the ubiquitin-proteasome pathway, respectively (Fig. 2) [3].

Fig. 2. HPV genes and their functions.



https://www.genpathdiagnostics.com/womens-health/gencerv/

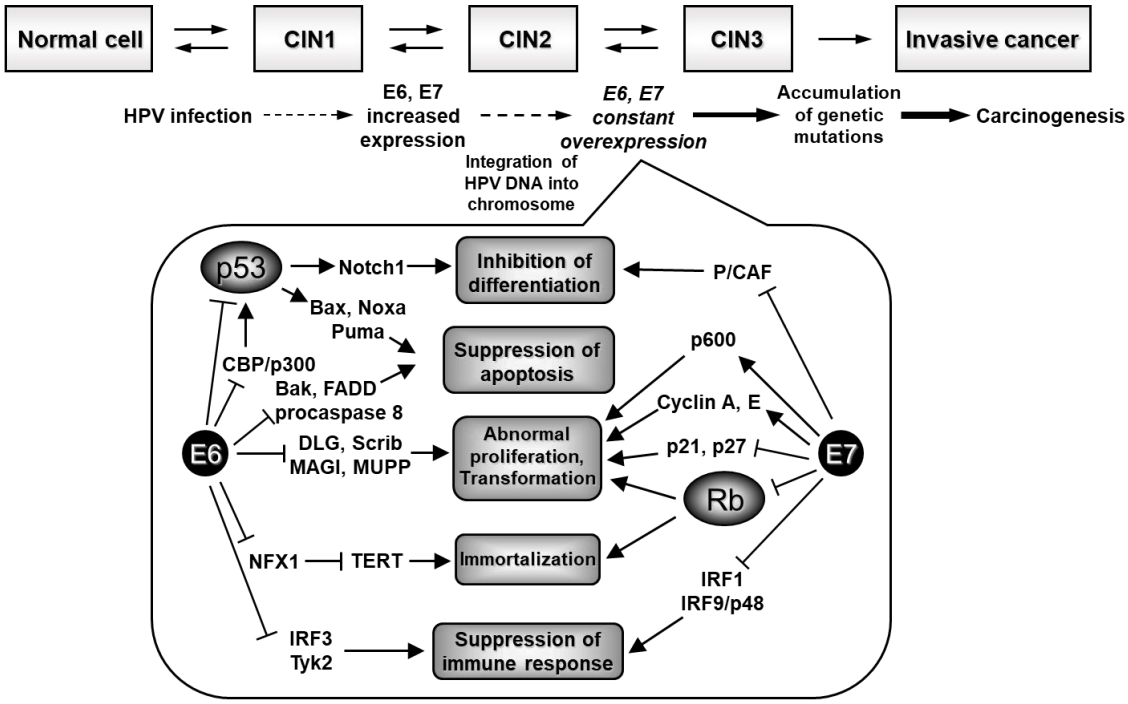


http://genetics.thetech.org/ask/ask359

Normal epithelial cells persistently infected with HR-HPV first develop low-grade CIN. When viral DNA is integrated into host chromosome, constant overexpression of E6 and E7 induces

abnormal proliferation, transformation and immortalization, and inhibits differentiation, apoptosis and immune response, leading to development of high-grade CIN. Accumulation of genetic and epigenetic alterations further causes progression to invasive cancer (Fig. 3) [4].

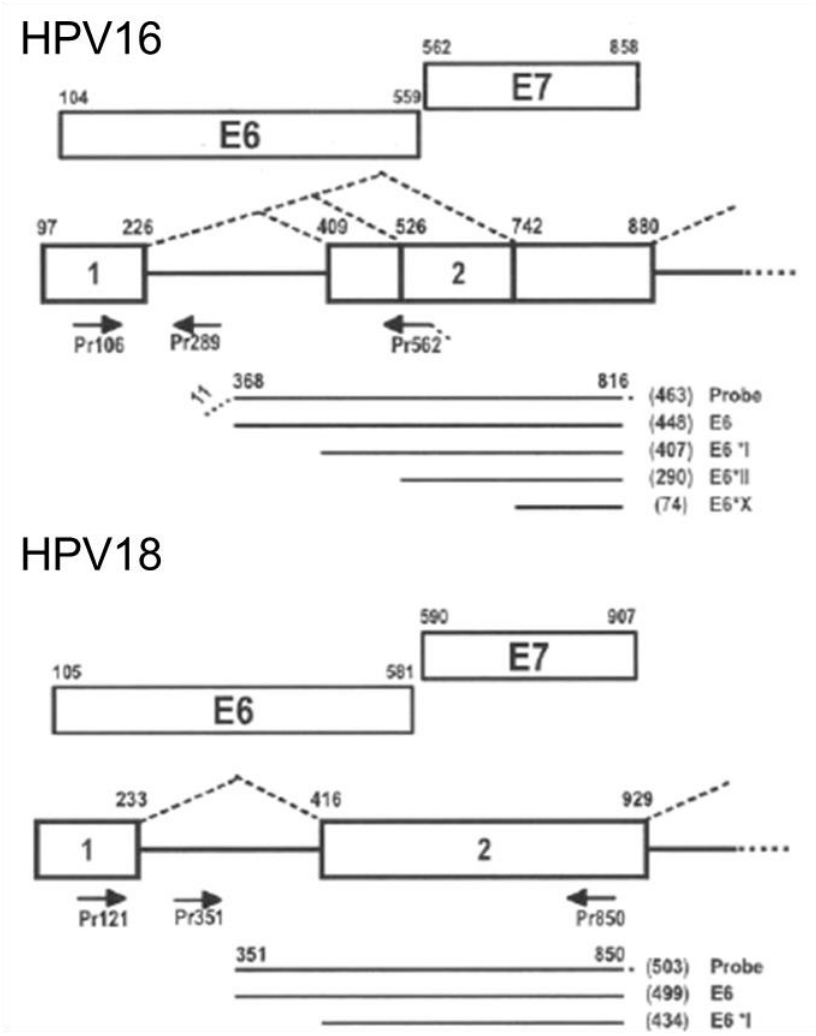
Fig. 3. Molecular mechanism of cervical carcinogenesis. [5]



Translation from Minaguchi T, et al. Gan To Kagaku Ryoho. 2010;37(1):18-22.

E6 is mainly expressed from full-length E6/E7 mRNA, and E7 is mainly expressed from spliced E6*/E7 mRNA (Fig. 4) [6].

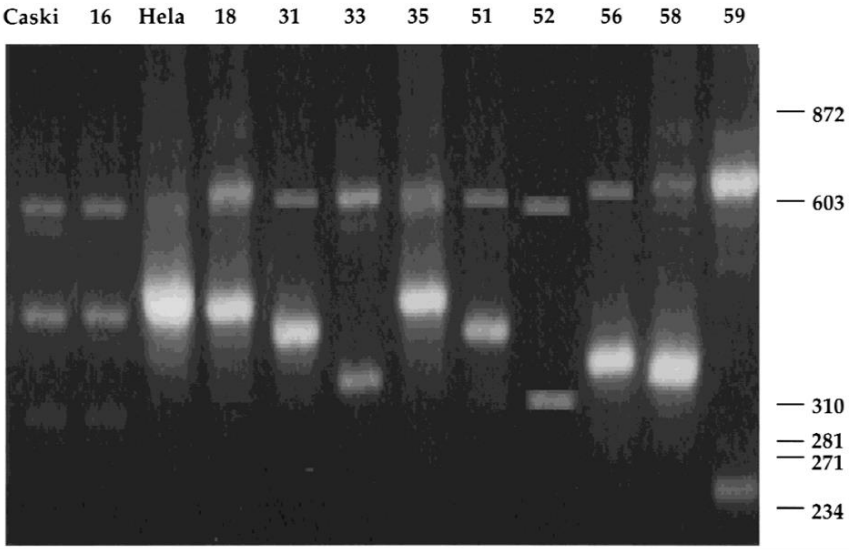
Fig. 4. Splicing patterns of E6 and E7 transcripts. [6]



Tang S et al. J Virol. 2006;80(9):4249-63

HPV-16 expresses two isoforms of E7 gene, and the other HPV types including HPV-18 express one isoform of E7 gene (Fig. 5).

Fig. 5. Detection of E6 and E7 mRNAs in cervical neoplasia samples and cervical cancer-derived cell lines. [7]



Nakagawa S, et al. J Med Virol. 2000;62(2):251-8.

To date, only a few studies previously investigated the distinct roles of E6 and E7 mRNAs for cervical carcinogenesis [7, 8]. According to Nakagawa et al., E6 transcript is uniformly detected from CIN1 to invasive cancer, but E7 transcripts show a higher detection rate with disease progression from low-grade CIN to invasive cancer (Fig. 6) [7].

Fig. 6. Detection of E6 and E7 mRNAs in cervical neoplasia samples and cervical cancer-derived cell lines. [7]

TABLE I. Detection of HPV E6 and E7 Transcripts in HPV-Positive Cervical Neoplasias

Lesion	HPV type										E6 transcript (%)	E7 transcript (%)
	16	18	31	33	35	51	52	56	58	59		
CIN I	1*							1	1*	1	4/4 (100%)	2/4 (50%)
CIN II	1*			1	1*			1	1		5/5 (100%)	3/5 (60%)
CIN III	6†		1*	2		1	2		2		14/14 (100%)	12/14 (86%)
Invasive cancer	13	8#	2	3	1		2		2		30/31 (97%)	31/31 (100%)

*Only E6 transcript was detected in each sample.
†Only E6 transcript was detected in one of 6 samples.
#Only E7 transcript was detected in one of 8 samples.

Nakagawa S, et al. J Med Virol. 2000;62(2):251-8.

Another previous publication by Sotlar et al. showed that detection rate of E7 transcript increased with disease progression in contrast with E6 transcript showing only moderate increase (Fig. 7) [8].

Fig. 7. Detection of HPV E6 and E7 transcripts by nested RT-PCR in cervical scrapes. [8]

			HR-HPV mRNA [n (%)]	
	Total	HR-HPV-DNA	E6/E7 ± E6*	E6*
CIN 0	294	80	27 (33.8)	14 (17.5)
CIN I	56	46	35 (76.1)	26 (56.5)
CIN II	64	60	54 (90.0)	46 (76.7)
CIN III	45	43	41 (95.3)	36 (83.7)
Total	459	229	157 (68.6)	122 (53.3)

Sotlar K, et al. J Med Virol. 2004;74(1):107-16.

The aim of our study was to investigate the distinct roles of each E6 and E7 mRNAs in the pathogenesis of cervical cancer [9].

2. MATERIALS AND METHODS

2.1 Patients and samples

The current study comprised two parts: a cross-sectional study of analyzing E6/E7 mRNAs in cervical specimens from patients with CIN or invasive cervical carcinoma and an adjunctive longitudinal study of following up patients with CIN1-2. Women with histologically and newly diagnosed CIN or cervical carcinoma were eligible to participate in this study and recruited between December 2014 and April 2017 at the outpatient clinic of University of Tsukuba Hospital. The study population was composed of CIN1 (n=16), CIN2 (n=33), CIN3 (n=83) and cervical carcinoma (n=39). The median age was 41.0 years for CIN1 (range 23-59), 33.0 years for CIN2 (range 22-65), 36.0 years for CIN3 (range 22-70), and 49.0 years for cervical carcinoma (range 33-76). Cervical specimens were collected with a Rovers Cervex-Brush (Rovers Medical Devices, Oss, The Netherlands) into a ThinPrep vial containing PreservCyt solution (HOLOGIC, Tokyo, Japan). Cells were immediately collected and stored in -80^{o}C until use. Study protocol was approved by the Ethics Committee University of Tsukuba Hospital (H26-119). Written informed consent was obtained prior to enrollment of participants. Histology was evaluated based on the most severe lesion present. Cytology was classified according to the Bethesda system [10]. The included patients were treated or followed-up according to the clinical guidelines [11]. Study results of the mRNA analyses did not influence their management. The median follow-up duration was 194 days (range 0-613). Follow-up data were retrieved until 2017-5-31.

2.2 DNA extraction and HPV genotyping

Genomic DNA was extracted using SepaGene kit (Eidia, Tokyo, Japan) according to the manufacturer's instruction. HPV genotyping was done by L1-PCR and RFLP analyses as described previously [7] or at a clinical testing laboratory (SRL, Tokyo, Japan) by Amplicor linear array HPV genotyping test (Roche Diagnostics, Tokyo, Japan). HR-HPVs are defined as HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68, which can be detected by Hybrid Capture 2 (HC2).

2.3 RNA extraction and Reverse Transcriptase PCR (RT-PCR)

Total RNA extraction and DNase treatment were performed as described previously [7]. RT-PCR was conducted using OneStep RT-PCR kit (QIAGEN, Tokyo, Japan) according to the manufacturer's instruction. We utilized a RT-PCR assay based on consensus primers designed to maintain around 80–90% homology to the known conserved sequences in E6 and E7 ORFs among multiple oncogenic HPVs [7]. E6 and E7 mRNAs could be separately detected for at least HPV types 16, 18, 31, 33, 35, 51, 52, 56, 58 and 59 [7]. We used β2-microglobulin as a control for RT-PCR in order to validate normal RNA extraction and no contamination of DNA which will affect the RT-PCR results, as E6/E7 DNA is the same size as E6 mRNA. Primers used for RT-PCR and PCR are as follows: E6/E7, ACC GAA AAC GGT TGA ACC GAA

AAC GGT and GAG CTG TCG CTT AAT TGC TC; β2-microglobulin, TGT CTT TCA GCA AGG ACT GG and GAT GCT GCT TAC ATG TCT CG.

2.4 Statistical analysis

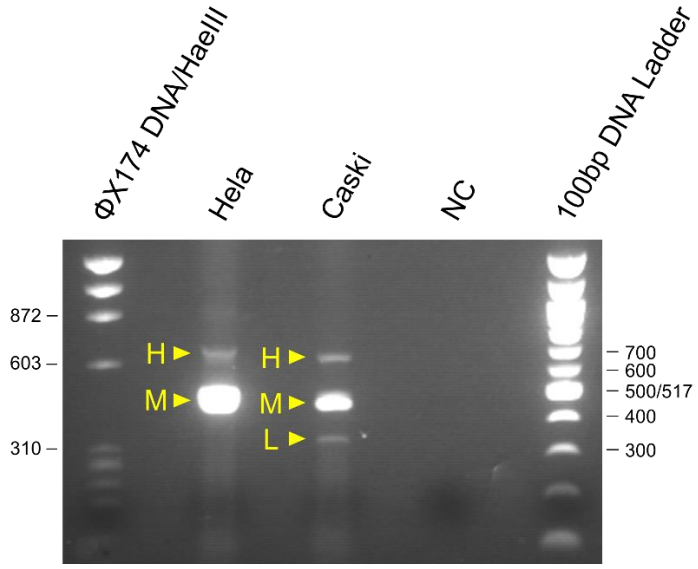
Differences in proportions were evaluated by the Fisher's exact test. Diagnostic indices of sensitivities, specificities, positive predictive values, and negative predictive values with 95% confidence intervals were calculated for detecting CIN2+, CIN3+, and invasive cervical cancer. Disease progression of CIN1-2 was examined as a surrogate by the Kaplan-Meier method calculating the intervals from E6/E7 sample collection until patients showed upgraded results of Pap test compared with the cytology at E6/E7 sample collection or they were censored, and the difference was statistically evaluated by the log-rank test.

3. RESULTS

We first analyzed the E6 and E7 mRNA expression patterns in human cervical cancer cell-lines CaSki and HeLa by RT-PCR (Fig. 8) [9], and confirmed that the expression patterns were consistent with data published by Nakagawa et al. [7]. In addition to E6 mRNA, two isoforms of E7 mRNA were detected in HPV 16-positive CaSki cells, and one isoform of E7 mRNA detected in HPV 18-positive HeLa cells. In order to verify that our RT-PCR assay works properly, we further performed sequencing analyses of E6/E7 cDNAs and confirmed that E6

mRNA is actually full-length E6/E7 transcript and that E7 mRNAs are actually spliced E6*/E7 transcripts (Fig. 9 and 10). The E6/E7 DNA is the same size as the full-length RNA, 652 bp for HeLa and 622 bp for CaSki. β2-microglobulin is 148 bp for RNA and 775 bp for DNA (Fig. 11) [9].

Fig. 8. E6/E7 mRNA expression patterns by RT-PCR in human cervical cancer cell lines, HeLa and CaSki. H: full-length E6/E7 (E6), M: spliced E6*I/E7 (E7), L: spliced E6*II/E7 (E7).



Liu S, et al. PLoS One. 2018;13(2):e0193061.

Fig. 9. Sequencing analyses of RT-PCR products of E6/E7 from CaSki cells.

CaSki E6/E7

```
>vg:1489078 E6, HpV16gp1; Human papillomavirus type 16; transforming protein
E6
Length=477

 Score =   852 bits (944),  Expect = 0.0
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 Strand=Plus/Plus

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Sbjct  421  GGTCGATGTATGTCTTGTTGCAGATCATCAAGAACACGTAGAGAAACCCAGCTGTAA  477
```

```
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E7
Length=297

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 Identities = 57/58 (98%), Gaps = 1/58 (2%)
 Strand=Plus/Plus

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```

CaSki E6*I/E7

```
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 Strand=Plus/Plus

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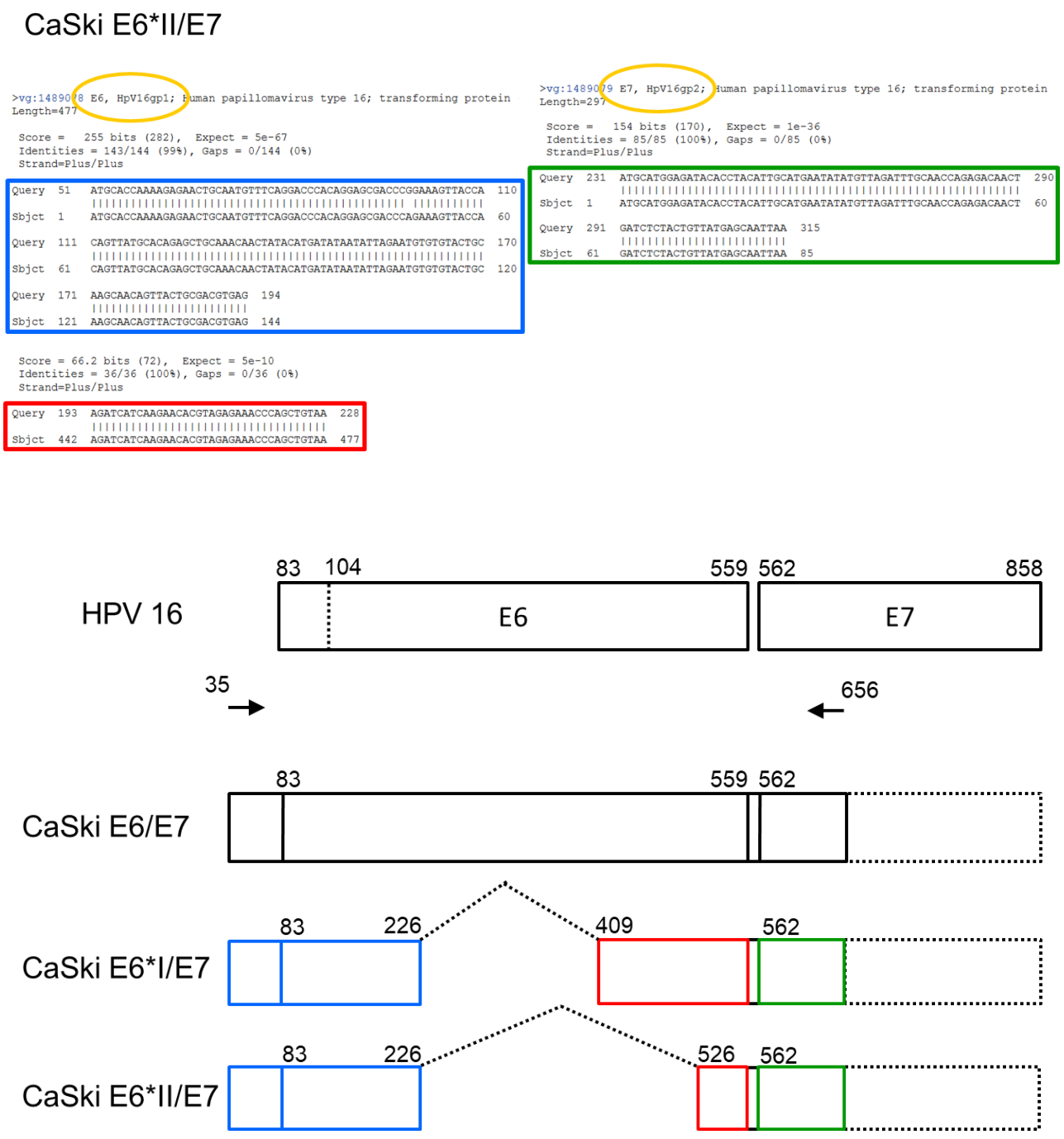

Fig. 10. Sequencing analyses of RT-PCR products of E6/E7 from HeLa cells.

HeLa E6/E7

```
>vg:1489083 E6, HpV18gp1; Alphapapillomavirus 7; E6 protein
Length=477

 Score =   810 bits (898),  Expect = 0.0
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 Strand=Plus/Plus

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HeLa E6*I/E7

```
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Length=477

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 Strand=Plus/Plus

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Query  256  AAACGACGATTCCACAACATAGCTGGGCACTATAGAGGCCAGTGCCATTCGTGCTGCAAC  315
            |||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
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Query  316  CGAGCACGACAGGAAAGACTCCAACGACGCAGAGAAACACAAGTATAA  363
            ||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct  430  CGAGCACGACAGGAACGACTCCAACGACGCAGAGAAACACAAGTATAA  477
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 Score =   237 bits (262),  Expect = 2e-61
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 Strand=Plus/Plus

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Sbjct  121  CTTACAGAGGT  131
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Length=318

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Query  432  ATTCCGGTTGACCTTCTATGTCACGAGCAATTAAGCGAC  470
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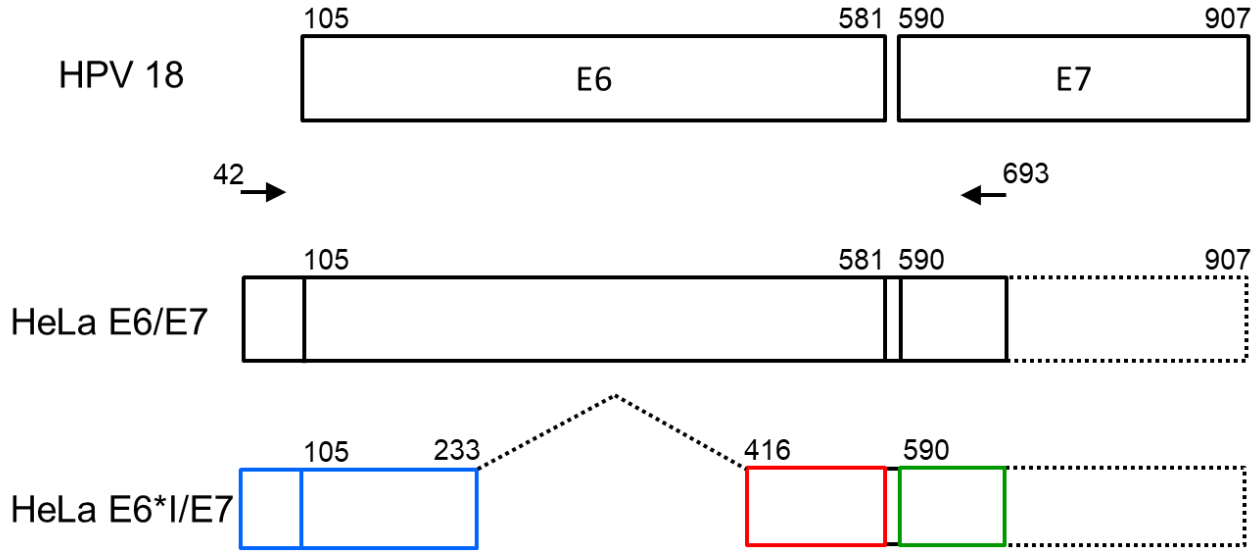
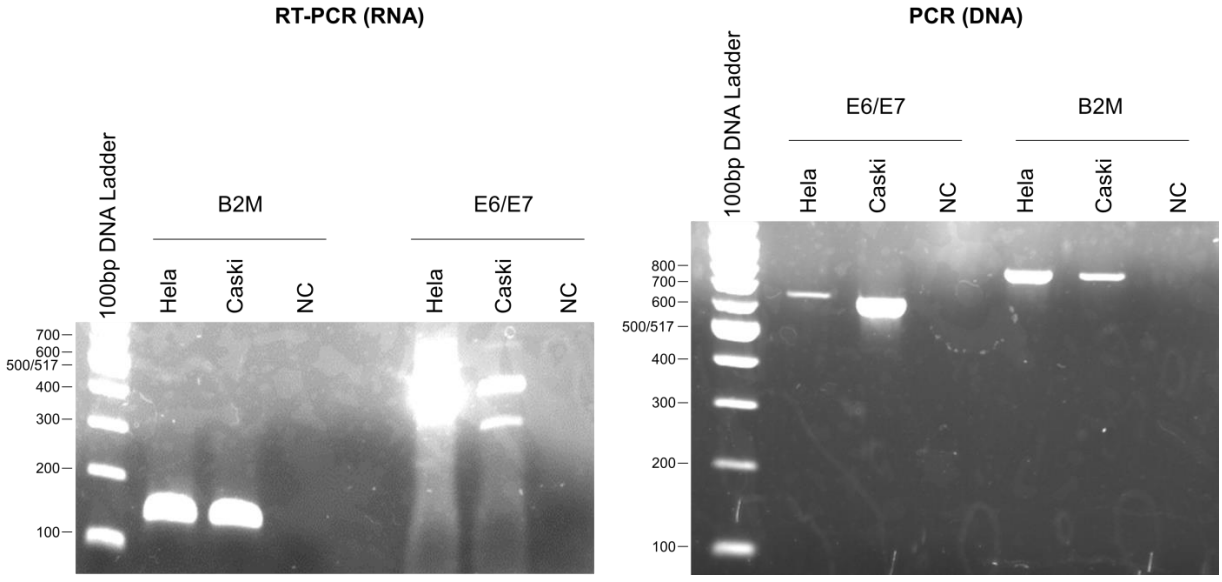


Fig. 11. Comparison between DNA and RNA of HPV E6/E7 and human β2-microglobulin (B2M) genes. The size of E6/E7 DNA is 652bp for HeLa and 622bp for CaSki, same as E6 mRNA. The size of B2M DNA is 775bp and B2M RNA is 148bp.



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Subsequently we analyzed the E6/E7 mRNA expressions in liquid-based cytology (LBC) samples from 171 patients. Fig. 12 shows an example of detection of E6/E7 mRNA in cytology samples from patients [9]. Beta2-microglobulin amplification showed no contamination by genomic DNA in every sample. The detection rate of E7 mRNA significantly increased with disease progression from low-grade CIN to invasive cancer, while those of E6 mRNA and high-risk HPV DNA did not change (p=0.00011, 0.80 and 0.54, respectively; Table 1) [9]. We next examined the relationship between E6/E7 mRNA expressions and HPV genotypes. Presence of E6 mRNA showed significant associations with positive HR-HPV DNA but not with positive HPV16/18 DNA (p=0.0036 and 0.089; Table 2) [9], whereas presence of both E6 and E7 mRNAs showed significant associations with positive HPV16/18 DNA but not with positive HR-HPV DNA (p=0.0079 and 0.21; Table 2) [9].

Fig. 12. Detection of E6/E7 mRNAs in LBC samples from patients. E6 is detected in samples 1 and 3, and 2 isoforms of E7 are detected in samples 2 and 3.

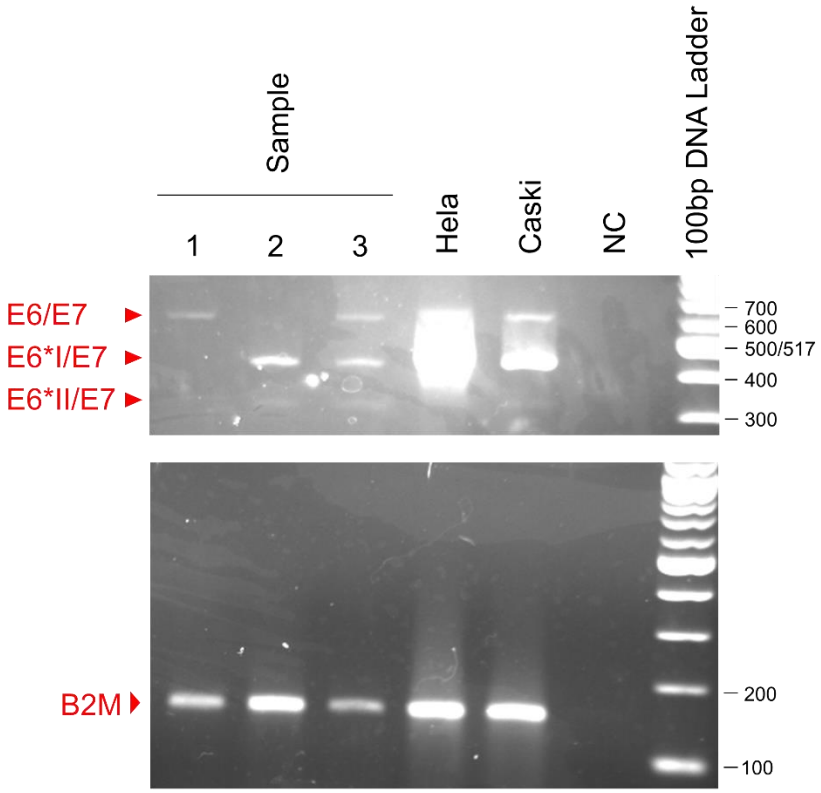

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Table 1. E6/E7 mRNA analyses and HPV genotyping in LBC samples from patients with cervical neoplastic diseases.

	CIN1	%	CIN2	%	CIN3	%	CxCa	%	*P*-value
E6 mRNA(+)	7/16	44	16/33	48	35/83	42	20/39	51	0.80
E7 mRNA(+)	1/16	6	4/33	12	20/83	24	21/39	54	0.00011
E6 mRNA(+) and E7 mRNA(+)	0/16	0	4/33	12	12/83	14	14/39	36	0.0047
E6 mRNA(+) and/or E7 mRNA(+)	8/16	50	16/33	52	43/83	52	27/39	69	0.27
High-risk HPV DNA(+)	13/16	81	30/33	91	75/83	90	33/39	85	0.54
HPV16/18 DNA(+)	2/16	13	12/33	36	36/83	43	25/39	64	0.030

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Table 2. Relationship between E6/E7 mRNAs and HPV genotypes.

	High-risk HPV DNA			HPV16/18 DNA		
	(+)	(−)	*P*-value	(+)	(−)	*P*-value
E6 mRNA(+)	75/151 (50%)	3/20 (15%)	0.0036	40/75 (53%)	38/96 (40%)	0.089
E7 mRNA(+)	40/151 (26%)	6/20 (30%)	0.79	26/75 (35%)	20/96 (21%)	0.056
E6 mRNA(+) and E7 mRNA(+)	29/151 (19%)	1/20 (5%)	0.21	20/75 (27%)	10/96 (10%)	0.0079
E6 mRNA(+) and/or E7 mRNA(+)	86/151 (57%)	8/20 (40%)	0.16	46/75 (61%)	48/96 (50%)	0.16

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Next, we examined diagnostic accuracies for detecting cervical neoplastic diseases by E6/E7 mRNA analyses. For detecting CIN2+, presence of both E6 and E7 mRNAs showed high specificity and low sensitivity (100% [95% confidence interval {CI}, 79-100] and 19% [95% CI, 13-26]; Table 3) in contrast with positivity of HR-HPV DNA showing high sensitivity and low specificity (89% [95% CI, 83-93] and 19% [95% CI, 4-46]; Table 3) [9]. Notably, the positive predictive value (PPV) for detecting CIN2+ was even higher by the presence of both E6 and E7 mRNAs than by the positivity for HR-HPV DNA or HPV16/18 DNA (100%, 91% and 91%, respectively; Table 3) [9]. Similar trends were also observed about the diagnostic accuracies for detecting CIN3+ and invasive cervical cancer (Tables 4 and 5) [9].

Table 3. Diagnostic indices of E6/E7 mRNA analyses for detecting CIN2+.

	Sensitivity (% [95% CI])	Specificity (% [95% CI])	PPV (% [95% CI])	NPV (% [95% CI])
E6 mRNA(+)	71/155 (46 [38-54])	9/16 (56 [30-80])	71/78 (91 [82-96])	9/93 (10 [5-18])
E7 mRNA(+)	45/155 (29 [22-37])	15/16 (94 [70-100])	45/46 (98 [88-100])	15/125 (12 [7-19])
E6 mRNA(+) and E7 mRNA(+)	30/155 (19 [13-26])	16/16 (100 [79-100])	30/30 (100 [88-100])	16/141 (11 [7-18])
E6 mRNA(+) and/or E7 mRNA(+)	86/155 (55 [47-63])	8/16 (50 [25-75])	86/94 (91 [84-96])	8/77 (10 [5-19])
High-risk HPV DNA(+)	138/155 (89 [83-93])	3/16 (19 [4-46])	138/151 (91 [86-95])	3/20 (15 [3-38])
HPV16/18 DNA(+)	73/155 (47 [39-55])	14/16 (88 [62-98])	73/75 (97 [91-100])	14/96 (15 [8-23])

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Table 4. Diagnostic indices of E6/E7 mRNA analyses for detecting CIN3+.

	Sensitivity (% [95% CI])	Specificity (% [95% CI])	PPV (% [95% CI])	NPV (% [95% CI])
E6 mRNA(+)	55/122 (45 [36-54])	26/49 (53 [38-67])	55/78 (71 [59-80])	26/93 (28 [19-38])
E7 mRNA(+)	41/122 (34 [25-43])	44/49 (90 [78-97])	41/46 (89 [76-96])	44/125 (35 [27-44])
E6 mRNA(+) and E7 mRNA(+)	26/122 (21 [14-30])	45/49 (92 [80-98])	26/30 (87 [69-96])	45/141 (32 [24-40])
E6 mRNA(+) and/or E7 mRNA(+)	70/122 (57 [48-66])	25/49 (51 [36-66])	70/94 (74 [64-83])	25/77 (32 [22-44])
High-risk HPV DNA(+)	108/122 (89 [81-94])	6/49 (12 [5-25])	108/151 (72 [64-79])	6/20 (30 [12-54])
HPV16/18 DNA(+)	61/122 (50 [41-59])	35/49 (71 [57-83])	61/75 (81 [71-89])	35/96 (36 [27-47])

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Table 5. Diagnostic indices of E6/E7 mRNA analyses for detecting invasive cervical cancer.

	Sensitivity (% [95% CI])	Specificity (% [95% CI])	PPV (% [95% CI])	NPV (% [95% CI])
E6 mRNA(+)	20/39 (51 [35-68])	74/132 (56 [47-65])	20/78 (26 [16-37])	74/93 (80 [70-87])
E7 mRNA(+)	21/39 (54 [37-70])	107/132 (81 [73-87])	21/46 (46 [31-61])	107/125 (86 [78-91])
E6 mRNA(+) and E7 mRNA(+)	14/39 (36 [21-53])	116/132 (88 [81-93])	14/30 (47 [28-66])	116/141 (82 [75-88])
E6 mRNA(+) and/or E7 mRNA(+)	27/39 (69 [52-83])	65/132 (49 [40-58])	27/94 (29 [20-39])	65/77 (84 [74-92])
High-risk HPV DNA(+)	33/39 (85 [69-94])	14/132 (11 [6-17])	33/151 (22 [16-29])	14/20 (70 [46-88])
HPV16/18 DNA(+)	25/39 (64 [47-79])	82/132 (62 [53-70])	25/75 (33 [23-45])	82/96 (85 [77-92])

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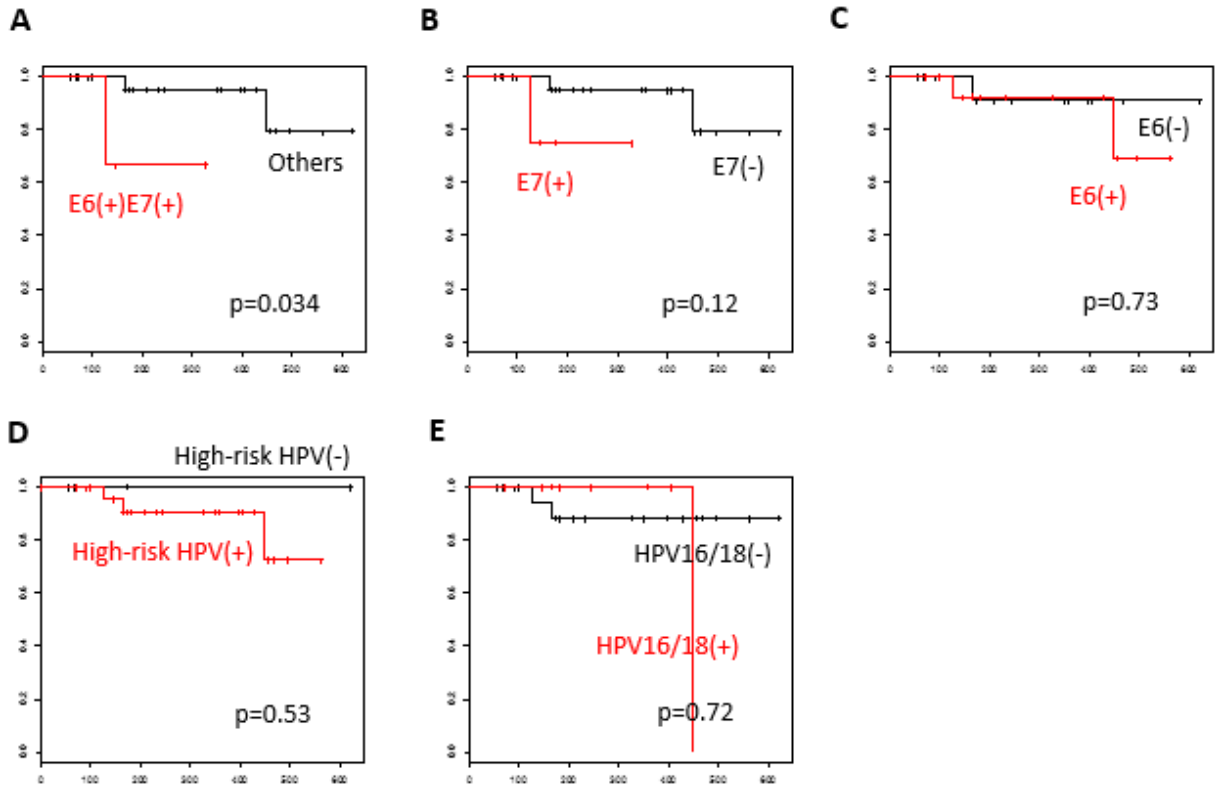
Finally, we examined the impact of presence of E6/E7 mRNAs or HPV genotypes on disease progression by following up 31 patients with CIN1-2. Since no disease progression was pathologically diagnosed yet in those patients, we compared intervals until the occurrence of upgraded abnormal cytology compared with the cytology at E6/E7 sample collection as a surrogate for disease progression. Presence of both E6 and E7 mRNAs showed significant association with the occurrence of upgraded abnormal cytology, presence of E7 mRNAs

showed association without statistical significance, but positive E6 mRNA, HR-HPV DNA, or HPV 16/18 DNA showed no such trends (p=0.034, 0.12, 0.73, 0.53, and 0.72, respectively; Fig. 13) [9].

Fig. 13. Kaplan-Meier curves for upgraded Pap-test results in followed-up patients with CIN1-2. *A*, cases positive for both E6 and E7 mRNAs (n=3) *vs.* the remainder (n=28); *B*, cases with positive E7 mRNAs (n=4) *vs.* negative E7 mRNAs (n=27); *C*, cases with positive E6 mRNA (n=15) *vs.* negative E6 mRNA (n=16); *D*, cases with positive HR-HPV DNA (n=26) *vs.* negative HR-HPV DNA (n=5); *E*, cases with positive HPV16/18 DNA (n=8) vs. negative HPV16/18 DNA (n=23).



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4. DISCUSSION

First of all, regarding novel points of our study based on the previous publications, our study is the first to investigate associations of positivity of E7 mRNA and positivity of both E6 and E7 mRNAs separately with disease progression and diagnostic indices in clinical samples. Our study is also the first to separately analyze E6 and E7 mRNAs on disease progression in follow-up patients.

Our E6/E7 RT-PCR analyses showed that E7 mRNAs were significantly associated with progression from low-grade CIN to invasive carcinoma in contrast with E6 mRNA showing no such trend (Table 1) [9]. Furthermore, we found that the presence of E6 mRNA was significantly associated with the positivity for HR-HPV DNA but not with the positivity for HPV16/18 DNA, whereas the presence of both E6 and E7 mRNAs was significantly associated with the positivity for HPV16/18 DNA but not with the positivity for HR-HPV DNA (Table 2) [9]. These observations suggest that E7 mRNA may be more closely involved in cervical carcinogenesis than E6 mRNA and that the presence of both E6 and E7 mRNAs may be the oncogenic property specific for HPV16/18, keeping in line with previous publications where the expression of E7 by itself can immortalize human keratinocytes at a low frequency but E6 has no such activity, and the combination of E6 and E7 is highly efficient at immortalizing most types of primary cells [12, 13]. Additionally in the transgenic mouse model, E7 alone, but not

E6 alone, is reported to be sufficient to induce high-grade CIN and invasive cervical cancers and the addition of E6 results in larger and more extensive cervical cancers [14].

Although E7 mRNA was most significantly associated with disease progression from low-grade CIN to invasive carcinoma in 171 patients (Table 1) [9], the positivity of both E6 and E7 mRNAs was the only significant factor in 31 patients of the follow-up study (Fig. 13) [9]. The reason for this discrepancy may be possibly that while positive E7 mRNA is most associated with the present status of cervical neoplastic disease, positivity of both E6 and E7 mRNA is most important for future disease progression. Previous publications only investigated single positivity of E6 or E7 mRNA, but not the positivity of both E6 and E7 mRNAs.

Oncoproteins E6 and E7 are known to cause development of cervical cancer by inactivating the tumor suppressors p53 and Rb, respectively. Accordingly, our above findings suggest that Rb may play a more critical role in cervical carcinogenesis than p53, being consistent with the published finding that Rb and Ki67 were the strongest predictive markers for CIN progression among various molecular markers including p53 [15]. P53 transcription factor is activated in response to stress signals and induces various biological functions including apoptosis, senescence, cell cycle arrest, DNA repair, and autophagy. Rb binds to E2F transcription factor, resulting in inhibition of cell cycle transition from G1 to S phase. However, the detailed mechanism whereby Rb plays a more important role in cervical carcinogenesis than p53 is

unknown yet. We will consider it as our future perspective, for example by using siRNA inhibiting Rb or p53. Moreover, E7 is known to bind to Rb and degrade it through the ubiquitin-proteasome pathway. E7 of high-risk HPVs reportedly has stronger affinity to Rb than that of low-risk HPVs [16]. However, the detailed mechanism on the E7 structure and function is also unknown yet. We will also consider this as our future perspective, for example by comparing and analyzing DNA sequences of E7 of various high-risk and low-risk HPVs.

Diagnostic indices by our E6/E7 RT-PCR analyses for detecting cervical neoplastic diseases showed that positivity of both E6 and E7 mRNAs had high specificity and low sensitivity in contrast with positive HR-HPV DNA having high sensitivity and low specificity (Tables 3-5) [9]. HC2 is indeed reported to show high sensitivity and relatively low specificity (88.8-95.8% and 38.7-56% for CIN2+) [17-20]. Notably, the PPV for detecting CIN2+ by the presence of both E6 and E7 mRNAs was even higher than by the positivity for HR-HPV DNA (Table 3) [9]. Accordingly, the separate analysis of E6 and E7 mRNAs may be more useful than HR-HPV test for detecting CIN2+ precisely. As with positive both E6 and E7 mRNAs, LBC test is also reported to have high specificity for detecting cervical neoplastic diseases (84.8-94.1% for CIN2+) [21]. However, while cytology test is considered to reflect the present status of diseases, E6/E7 mRNA analysis may be able to predict future disease progression, as this test examines HPV oncogene expressions with transforming abilities. In this context, we further examined the

impact of presence of E6/E7 mRNAs on disease progression by following up patients with CIN1-2. Presence of both E6 and E7 mRNAs showed significant associations with the occurrence of upgraded abnormal cytology, presence of E7 mRNA showed association without statistical significance, while positive E6 mRNA, HR-HPV DNA, or HPV 16/18 DNA showed no such trends (Fig. 13) [9]. Regarding follow-up study of HPV mRNA tests, the longitudinal studies have reported that positive mRNA at baseline is an excellent predictor for future development of CIN2+ or CIN3+ in referral or post-treatment populations [22-28]. Together with these published findings, our above observations suggest that the separate analysis of E6 and E7 mRNAs may predict disease progression of CIN more precisely than HPV DNA tests. However, further following up patients and pathologically detecting disease progression are required to clarify the predictive significance of separately analyzing E6 and E7 mRNAs.

The sensitivity of our E6/E7 mRNA test for detecting CIN2+ is lower than those of other reported HPV RNA tests (77.0-96.3% for CIN2+) [17-20, 25, 29]. However, while almost all other HPV RNA tests examine E6 and E7 mRNAs collectively, our RT-PCR system can detect each E6 and E7 mRNAs separately so that disease progression may be more precisely predicted by individually evaluating E7 mRNA which seems more closely involved in cervical carcinogenesis than E6 mRNA. However, in order to improve the sensitivity of our separate E6/E7 mRNA analysis, it may be necessary to try changing our method to conventional 2-step

RT-PCR or the nested RT-PCR. We will also consider quantitative E6/E7 mRNA analysis by real-time RT-PCR for our future perspective, as it may reduce the frequency of false negative results.

In conclusion, our separate analyses of E6/E7 mRNAs demonstrated here that the presence of E7 mRNAs was significantly associated with progression from low-grade CIN to invasive carcinoma in contrast with positive E6 mRNA or HR-HPV DNA. Besides, the presence of both E6 and E7 mRNAs was significantly associated with the positivity for HPV16/18 DNA, while the presence of E6 mRNA was significantly associated with the positivity for HR-HPV DNA. The presence of both E6 and E7 mRNAs showed high specificity and low sensitivity for detecting CIN2+ by contrast with the positivity for HR-HPV DNA. Furthermore, the presence of both E6 and E7 mRNAs showed significant association with the occurrence of upgraded abnormal cytology in the patients followed-up for CIN1-2 by contrast with positive E6 mRNA, HR-HPV DNA, or HPV16/18 DNA. Our findings suggest a closer involvement of E7 mRNAs than E6 mRNA in cervical carcinogenesis, and the presence of both E6 and E7 mRNAs may exert the strongest transforming ability. Moreover, the separate analysis of E6 and E7 mRNAs may be a more useful tool than HR-HPV DNA test for detecting CIN2+ precisely and predicting disease progression. Further accumulation of evidence is warranted to validate our proposal.

5. ACKNOWLEDGMENTS

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The authors have no conflicts of interest to disclose.

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