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Original Paper

Telomerase Activity and Human Papillomavirus (HPV) Infection in Human Uterine Cervical Cancers and Cervical Smears

K. Kawai, Y. Yaginuma, H. Tsuruoka, M. Griffin, H. Hayashi and M. Ishikawa

Department of Obstetrics and Gynaecology, Asahikawa Medical College, 4–5 Nishikagura, Asahikawa 078, Japan

Telomerase activity and human papillomavirus (HPV) infection were investigated in uterine cervical samples using molecular biology techniques. Thirteen cervical carcinomas and corresponding normal tissue from the same patient, and 102 cervical swabs were examined. Telomerase activity was detected in 12 of 13 cervical cancer tissues (92%). Of the 12 cases that showed telomerase activity, all were HPV positive, and the one case that did not show telomerase activity was HPV negative. A telomeric repeat amplification protocol assay detected telomerase activity in one out of seven normal cervical tissues (14%), and this one case was HPV positive. In cervical smear samples, telomerase activity was detected in two out of 36 normal smears (6%; both HPV positive), in 10 of 32 (31%) CIN1 (cervical intraepithelial neoplasia) cases (three HPV positive), in four of five (80%) CIN2 cases (two HPV positive), in 15 of 21 (71%) CIN3 cases, (seven HPV positive) and in seven of eight (88%) squamous cell carcinoma cases (six HPV positive). These results suggest that telomerase activity may play some role in cervical carcinogenesis, and telomerase activity is associated with HPV infection in uterine cervical lesions. © **1998 Elsevier Science Ltd. All rights reserved.**

Key words: telomerase, HPV, cervix, cancer, smear
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INTRODUCTION

GENETIC ANALYSIS of human tumours strongly suggests that a number of genetic defects accumulate over time and combine to bring about the disruption of growth control that finally results in malignancy. Despite their prevalence, gynaecological tumours have only recently become a focus for molecular analysis [1–4]. Telomerase is a ribonucleoprotein enzyme capable of extending chromosome ends with specific telomeric DNA sequences [5]. This enzyme compensates for the end replication problem and allows cells to proliferate indefinitely [6]. Telomerase activity is present in the germline, and is found in most human cancer tissues [7–10].

The molecular genetics of cervical cancer has not yet been thoroughly assessed, despite the fact that this is a very prevalent and often fatal disease worldwide. Human papillomavirus (HPV) has been shown, by epidemiological and experimental studies, to be closely related to the development of genital warts, cervical intraepithelial neoplasia (CIN), and cervical cancer [11]. Certain cervical cancers have been shown to express HPV genes. The viral gene regulator E6 of HPV has been shown to bind p53 protein, and this association is thought to inactivate the normal functions of p53 [12, 13]. Also, the E7 protein of HPV has been shown to inactivate the *Rb* gene [14]. Quite recently it was also shown that the E6 protein of HPV type 16 induced telomerase activity *in vitro* [15]. Therefore, to confirm this fact in clinical samples, telomerase activity and HPV infection were analysed in cervical cancer tissues and cervical smears.

Correspondence to Y. Yaginuma.
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MATERIALS AND METHODS

Cell lines

Seven cervical carcinoma cell lines (Caski, SiHa, HT-3, C-33A, C-41, ME-180, and HeLa S-3) were used. We received all cell lines from the American Type Culture Collection (Rockville, Maryland, U.S.A.). All cell lines were maintained under conditions recommended by the suppliers.

Tissue samples

Tissues samples, frozen at − 80°C, were obtained from the Asahikawa Medical College Hospital through surgical operations. As cervical tumours, 11 squamous cell carcinomas, and

two adenocarcinomas were used. Thirteen normal cervical tissues from the same patients were used as controls. Clinical stages were Ia:2, Ib:6, IIb:3 and IIIb:2 (FIGO classification).

Cervical smears

The classification of cervical cytology [16] was performed according to WHO classification (dysplasia/CIN system). One hundred and two cervical smears were analysed for normal, premalignant lesions and cancer (negative:36, CIN1:32, CIN2:5, CIN3:21 and SCC:8). For cytological analysis and HPV detection, two cervical scrapes were taken using a scraper, as described previously [17]. The first scrape was used for routine cytomorphological examination. A second sample was taken in the same manner and immersed into a plastic container with distilled water, which was later submitted for DNA and telomerase extraction.

DNA and RNA extraction

High molecular weight genomic DNAs were extracted using a published proteinase K/phenol protocol [18], and total RNA was extracted using the guanidinium thiocyanate extraction procedure [19]. Extracted RNA was treated with RNase free-DNaseI under conditions recommended by the suppliers (Boehringer Mannheim, Indianapolis, U.S.A.).

Detection of HPV infection

Firstly, universal primers were used to screen HPV positive cases by using L1C1 (CGTAAACGTTTTCCCTATTTTTTT) and L1C2 (GTTATGTCGCATAAATCCCAT) primers. These primers amplified HPV 6, 11, 16, 18, 31, 33, 42, 52 and 58. For positive cases detected using L1C1 and L1C2 primers, HPV types 16 and 18 were determined using type specific primers as follows. HPV type 16 sense primer: GCAACCAGAGACAACTGATC, antisense primer: ATTGTAATGGGCTCTGTCCG, HPV type 18 sense primer: TCACGAGCAATTAAGCGACT, antisense primer: CTGAGCTTTCTACTACTAGC [20, 21]. HPV types 16 and 18 were classified because these types of HPV are considered high risk types. We considered that HPV types 16 and 18 were high risk type HPV and other types were non-high risk types.

Successful amplification of the β-globin fragment indicated that the sample was adequate for HPV analysis [22]. The entire polymerase chain reaction (PCR) sample was electrophoresed through a 1.5% agarose gel and 4% NuSieve agarose gel, then stained with ethidium bromide and the PCR products visualised under ultraviolet light.

Telomerase assay

Cell and tissue extracts were prepared and assayed using a PCR based telomerase assay (TRAP; telomeric repeat amplification protocol) as described previously [7]. All samples were washed in ice-cold phosphate buffered saline (PBS), then homogenised in 20–200 μl of 3-((3-cholamidopropyl)-dimethyl-ammonio)-1-propanesulphonate (CHAPS) lysis buffer [10 mM Tris–HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM ethyleneglycolamino ethyl tetraacetic acid (EGTA), 0.1 mM phenylmethylsulphonyl fluoride (PMSF), 5 mM β-mercaptoethanol, 0.5% CHAPS, 10% glycerol] depending on the size of the sample, by using a Kontes homogeniser. After 30 min of incubation on ice, the lysate was centrifuged at 14 000 ***g*** for 30 min at 4°C. The supernatant was aliquoted, flesh frozen in liquid nitrogen, and stored at −80°C. The protein concentration was determined using the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, Illinois, U.S.A.). The typical protein concentration used for the TRAP assay was 6 μg of tissues and 1 μg of cervical smear samples. The TRAP assay was performed as follows. For tissues, 6 μg of protein was assayed in 50 μl of reaction mixture, and for cervical smears, 1 μg of protein was assayed in 10 μl reaction mixture containing 20 mM Tris–HCl (pH 8.3); 1.5 mM $MgCl_2$; 63 mM KCl; 0.005% Tween-20; 1 mM EGTA; 50 mM dGTP, dATP, dTTP, dCTP; TS primer; T4g32protein; 2 U Taq polymerase (Perkin-Elmer Corp., Branchburg, New Jersey, U.S.A.). After 10 min of incubation at room temperature to allow telomerase-mediated extension of the TS primer, CX primer was added to the reaction tube, then subjected to 30 PCR cycles for tissues and 32 PCR cycles for cervical smears at 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min. ITAS (internal TRAP assay standard, obtained from Dr Shay, Dallas, Texas, U.S.A.) was used as a positive control. The PCR mixture (5 μl) was analysed on an 8% non-denaturing polyacrylamide gel. The gels were stained with SYBR Green I (FMC Bio Products, Rockland, Maine, U.S.A.). Extracts were diluted and analysed at four different concentrations (1-, 10-, 100- and 1000-fold dilutions) to determine the level of telomerase present and to avoid false negatives due to possible PCR inhibitors in the extract. If the TRAP assay revealed a positive result in the 1- and/or 10-fold dilutions, this was defined as low activity. If the TRAP assay revealed a positive result in the 100- and/or 1000-fold dilutions, this was defined as high activity.

Human telomerase RNA (hTR) expression

To determine the hTR levels in cervical lesions, reverse transcription–PCR (RT–PCR) was used. Complementary DNA was generated from total RNA using 200 units of the Moloney strain of murine leukaemia virus reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, Maryland, U.S.A.) with oligo(dT) as a primer. A PCR based on an existing protocol [23] was then carried out using the following sense and antisense primers. Sense primer: 5′TGTGAGCCGAGTCCTGGGTGCACG3′. Antisense primer: 5′TTTGTCTAACCCTAACTGAGAAGG3′. Successful amplification of the β-actin fragment indicated that the sample was adequate for analysis [24].

RESULTS

Telomerase activity and hTR expression in cervical cancer cell lines

All cell lines studied demonstrated high telomerase activity. hTR was expressed in all cell lines. In these cell lines, HPV status has already been reported [25, 26], and those results were confirmed by PCR. HPV type 16 was detected in SiHa and Caski, and type 18 was detected in ME-180, C-4I and HeLa S-3 cell lines.

Telomerase activity, hTR expression and HPV infection in tissue samples

In the present study, 26 tissue samples (normal cervix: 13, squamous cell carcinoma:11, adenocarcinoma:two) were analysed. In normal cervix, telomerase activity was detectable in only one of 13 cases (case 4, low activity:8%), and this case showed non-high risk HPV infection. hTR expression was detectable in 85% (11 of 13) of normal cervix by using RT–PCR. As shown in Table 1, in squamous cell carcinoma of the cervix, telomerase activity was detectable (Figure 1) in 10 of 11 cases (91%; low activity:55%, high activity:36%), all 10

Table 1. *Telomerase activity, hTR expression, and human papillomavirus (HPV) status in cervical lesions*

				Telomerase positive HPV +			
Case	Pathology	Stage	hTR	Type 16	Type 18	Others	Telomerase negative HPV –
1	SCC	Ia	+	L			
	normal		+				–
2	SCC	Ia	+			L	
	normal		+				–
3	SCC	Ib	+			L	
	normal		–				–
4	SCC	Ib	+	L			
	normal		+			L	
5	SCC	Ib	+			L	
	normal		+				–
6	SCC	Ib	+	H			
	normal		+				–
7	SCC	IIb	+				–
	normal		+				–
8	SCC	IIb	+	H			
	normal		+				–
9	SCC	IIb	+	H			
	normal		+				–
10	SCC	IIIb	+	L			
	normal		+				–
11	SCC	IIIb	+	H			
	normal		+				–
12	Adenocarcinoma	Ib	+				
	normal		+				–
13	Adenocarcinoma	Ib	+				
	normal		–				–

H, high activity; L, low activity; hTR, human telomerase RNA; SCC, squamous cell carcinoma.

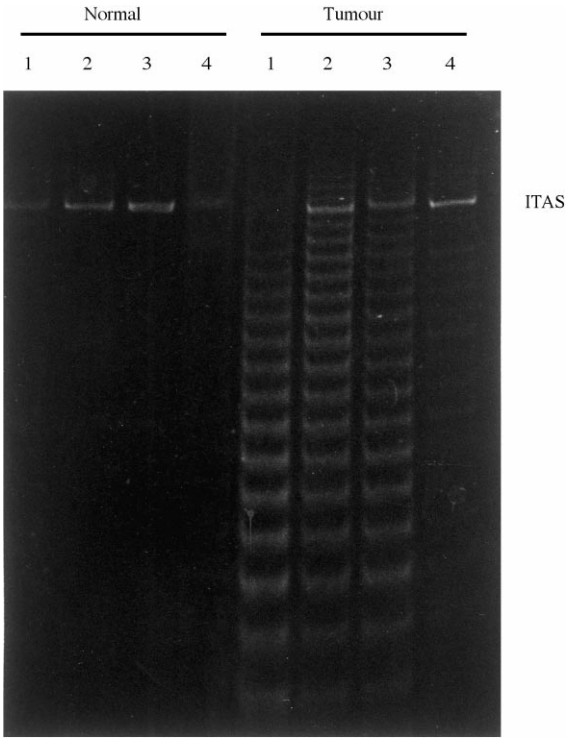

Figure 1. Telomerase activity in human cervical cancer using the TRAP assay. Lane 1, 6 μg; lane 2, 0.6 μg; lane 3, 0.06 μg; and lane 4, 0.006 μg protein concentration. The cervical cancer sample demonstrated a high level of telomerase activity, normal cervical tissue did not show any telomerase activity. The internal TRAP assay standard (ITAS) is clearly identified.

cases were HPV positive (high risk HPV: seven, non-high risk HPV:three). Clinical stages are shown in Table 1. Both stage Ib adenocarcinomas showed telomerase activity (one high activity, one low activity), and these two cases showed high risk HPV. In cases of high risk HPV, a high level of telomerase activity was detected in five out of nine cases (56%). Only low telomerase activity was detected in three cases of non-high risk HPV.

In total, telomerase activity was detected in 12 of 13 cases (92%) of cervical cancer. hTR expression was detectable in all cases of cervical cancer (Figure 2).

Telomerase activity and HPV infection in cervical smears

Technically it is very difficult to extract protein and nucleic acid from premalignant lesions, because such lesions cannot be identified macroscopically, even though in most cases a colposcope is used. Therefore, cervical smears were used to analyse premalignant lesions of the cervix.

As shown in Table 2, telomerase activity was detected in 6% (two of 36) of normal cases, 31% (10 of 32) of CIN1, 80% (four of five) of CIN2, 71% (15 of 21) of CIN3, and 88% (seven of eight) of squamous cell carcinoma. Telomerase activation can occur at any stage from normal tissue to cancer.

In normal cervical smears, HPV was detectable in two out of 36 cases (6%) (Table 2). One was high risk HPV, and the other was non-high risk type.These two cases showed telomerase activity. Telomerase activity was not detected in any normal HPV negative cases.

In CIN1 cases, telomerase activity was detected in three out of seven (43%) HPV positive cases , one high risk HPV and two non-high risk type. Seven of 25 HPV negative cases

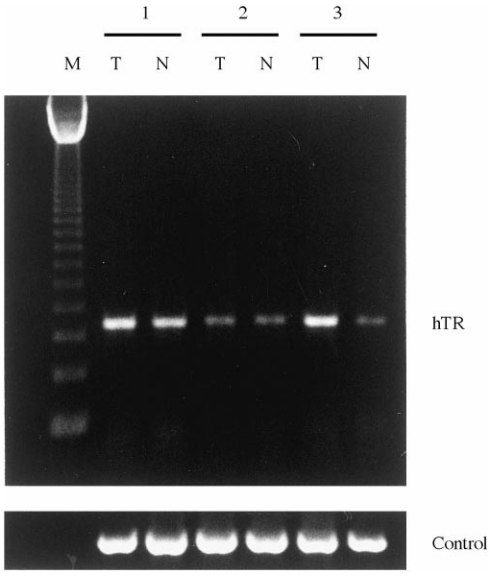

Figure 2. hTR expression by reverse transcription–polymerase chain reaction (RT–PCR) in cervical lesions. M, molecular weight marker (123 bp marker). T, tumour tissue; N, normal tissue. In three tumour cases, hTR was amplified. Also, all three normal cervical tissues showed hTR expression, but one of three cases revealed low expression compared with cancer tissue. β-actin was used as a control.

(28%) showed telomerase activity. In CIN2 cases, telomerase activity was detected in two out of two HPV positive cases (100%), one high risk HPV, and the other non-high risk type. Two of three HPV negative cases showed telomerase activity. In CIN3 cases, telomerase activity was detected in seven out of 11 HPV positive cases (64%), three high risk HPV, and four non-high risk HPV. Eight of 10 HPV negative cases showed telomerase activity (80%).

In squamous cell carcinoma cases, telomerase activity was detected in six out of seven cases (86%), four high risk HPV and two non-high risk type. In this group, both HPV types 16 and 18 were found in two cases. Telomerase activity was detected in one HPV negative sample.

DISCUSSION

This is the first study to analyse telomerase activity, HPV infection and hTR expression in cervical lesions. Telomerase consists of a protein component and an RNA component. This RNA component is called hTR. Eighty-five per cent of normal cervix were positive for hTR expression, whilst telomerase activity was detectable in only 8%. Also, hTR expression was detectable in all cases (100%) of cervical cancer, and telomerase activity was detected in 92% (12 of 13). It was surprising that hTR was expressed in normal tissues that lacked telomerase activity. In both normal and cervical cancer, hTR expression was detected more frequently than telomerase activity. This anomaly could be because hTR expression alone is not sufficient to produce telomerase activity, and there may be a complicated activation mechanism that is still unknown. Another reason could be that the TRAP assay is not sensitive enough to detect very low telomerase activity in normal cervix.

Associations between telomerase activity and disease stage have been reported previously in human laryngeal cancer, neuroblastoma, and gastric cancer [8, 27]. In our study, the clinical sample was too small to determine any correlation between telomerase activity and clinical stage. For premalignant lesions of cervix, telomerase activity was detected in 6% (two of 36) of normal cases, 31 (10 of 32) of CIN1, 80% (four of five) of CIN2, 71% (15 of 21) of CIN3, and 88% (seven of eight) of squamous cell carcinomas. The onset of telomerase activation can vary considerably from normal tissue to cancer. Not all malignant and premalignant lesions exhibited telomerase activity. This suggests that telomerase activity may be required for most, but not all, cancers. In cervical lesions, there seem to be two mechanisms of telomerase activation: one associated with HPV infection, and the other, like other cancers, not associated with HPV infection. The results suggest that association between HPV infection and telomerase activation is more likely in cervical carcinogenesis and even in normal tissue.

Quite recently it has been shown that the E6 protein of HPV type 16 induces telomerase activity *in vitro* [15]. In the present studies, in cervical cancer tissues, high telomerase activity was detected in 56% (5 of 9) of patients with high risk HPV, and low telomerase activity was detected in the three cases of non-high risk HPV. We speculate that the level of telomerase activation may depend on HPV type. These data support the close relationship between HPV infection and telomerase activity in clinical samples.

Not all premalignant lesions will convert to cancer; some lesions are self-limited, whereas others may persist for years before malignant transformation. Thus, it is desirable to identify some markers that would be useful for the detection of precancerous lesions. Telomerase activity may serve as a marker for cancer risk assessment in patients with normal and premalignant cervical lesions, because it is detectable in premalignant lesions and even in normal cervical lesions with HPV infection. However, it will be important to determine in future studies whether telomerase positive normal tissue could change to premalignancy or even malignancy. Similarly, it will be important to follow whether telomerase positive CIN1 or 2 lesions could change to malignancy.

Table 2. Telomerase activity and human papillomavirus (HPV) status in cervical smears

	HPV +		HPV –	
Class	Telomerase positive	Telomerase negative	Telomerase positive	Telomerase negative
Normal ($n = 36$)	2	0	0	34
CIN1 ($n = 32$)	3	4	7	18
CIN2 ($n = 5$)	2	0	2	1
CIN3 ($n = 21$)	7	4	8	2
SCC ($n = 8$)	6	1	1	0
Total ($n = 102$)	20/29 (69%)	9/29 (31%)	8/73 (25%)	55/73 (75%)

SCC, squamous cell carcinoma.

In conclusion, telomerase activity may play some role in uterine cervical carcinogenesis, and telomerase activity is associated with HPV infection in cervical lesions. This is the first study to analyse telomerase activity, HPV infection and hTR expression in uterine cervical lesions.

1. Yaginuma Y, Yamashita K, Kuzumaki N, Fujita M, Shimizu T. ras Oncogene product p21 expression and prognosis of human ovarian tumors. *Gynecol Oncol* 1992, **46**, 45–50.
2. Yaginuma Y, Westphal H. Abnormal structure and expression of the p53 gene in human ovarian carcinoma cell lines. *Cancer Res* 1992, **52**, 4196–4199.
3. Yaginuma Y, Yamashita T, Takuma N, Katayama H, Ishikawa M. Analysis of the p53 gene in human choriocarcinoma cell lines. *Br J Cancer* 1995, **71**, 9–12.
4. Yaginuma Y, Katayama H, Kawai K, Duenas JC, Ishikawa M. Analysis of the retinoblastoma gene in human endometrial carcinoma. *Obstet Gyneco* 1996, **87**, 755–759.
5. Blackburn EH. Structure and function of telomerase. *Nature (Lond.)* 1991, **350**, 569–573.
6. Feng J, Funk WD, Wang S, *et al.* The RNA component of human telomerase. *Science (Washington DC)* 1995, **269**, 1236–1241.
7. Kim NM, Piatyszek MA, Prowse KR, *et al.* Specific association of human telomerase activity with immortal cells and cancer. *Science (Washington DC)* 1994, **266**, 2011–2015.
8. Hiyama E, Yokoyama T, Tatsumoto N, *et al.* Telomerase activity in gastric cancer. *Cancer Res* 1995, **55**, 3258–3262.
9. Chadeneau C, Hay K, Hirte H, Gallinger S, Bacchetti S. Telomerase activity associated with acquisition of malignancy in human colorectal cancer. *Cancer Res* 1995, **55**, 2533–2536.
10. Tahara H, Nakanishi T, Kitamoto M, *et al.* Telomerase activity in human liver tissue; comparison between chronic liver disease and hepatocellular carcinomas. *Cancer Res* 1995, **55**, 2734–2736.
11. Hall S, Lorincz A, Shah F, *et al.* Human papillomavirus DNA detection in cervical specimens by hybrid capture: correlation with cytologic and histologic diagnoses of squamous intraepithelial lesions of the cervix. *Gynecol Oncol* 1996, **62**, 353–359.
12. Werness BA, Levine AJ, Howley PM. Association of human papillomavirus type 16 and 18 E6 proteins with p53. *Science (Washington DC)* 1990, **248**, 76–79.
13. Yaginuma Y, Westphal H. Analysis of the p53 gene in human uterine carcinoma cell lines. *Cancer Research* 1991, **51**, 6506–6509.
14. Dyson N, Howley P, Munger K, Harlow E. The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science* 1989, **242**, 934–937.
15. Klingelhutz AJ, Foster SA, McDougall JK. Telomerase activity by the E6 gene product of human papillomavirus type 16. *Nature* 1996, **380**, 79–82.
16. Berek JS, Hacker NF, eds. *Practical Gynecologic Oncology*, 2nd edn. Baltimore, Williams & Wilkins, 1994, 205.
17. Rozendaal L, Walboomers JMM, van der Linden JC, *et al.* PCR based high-risk HPV test in cervical cancer screening gives objective risk assessment of women with cytomorphologically normal cervical smears. *Int J Cancer* 1996, **68**, 766–769.
18. Maniatis T, Fritsch EF, Sambrook J. Isolation of high-molecular weight DNA from mammalian cells. In Ford N, Nolan C, Ferguson M, eds. *Molecular Cloning: A Laboratory Manual*, Vol. 1. Cold Spring Harbor, New York, Cold Spring Harbor Laboratory, 1989.
19. Ausubel FM, Brent R, Kingston RE, *et al.* Preparation and analysis of RNA. In *Current Protocols in Molecular Biology*, Vol. 1. New York, Wiley Interscience, 1989, 4.0.1–4.10.8.
20. Yoshikawa H, Kawana T, Kitagawa K, Mizuno M, Yoshikura H, Iwamoto A. Amplification and typing of multiple cervical cancer-associated human papillomavirus DNAs using a single pair of primers. *Int J Cancer* 1990, **45**, 990–992.
21. Yoshikawa H, Kawana T, Kitagawa K, Mizuno M, Yoshikura H, Iwamoto A. Detection and typing of multiple genital human papillomaviruses by DNA amplification with consensus primers. *Jpn J Cancer Res* 1991, **82**, 524–531.
22. Bauer HM, Greer CE, Chambers JC, *et al.* Genital human papillomavirus infection in female university students as determined by a PCR-based method. *JAMA* 1991, **265**, 472–477.
23. Savoysky E, Yoshida K, Ohtomo T, *et al.* Down-regulation of telomerase activity is an early event in the differentiation of HL60 cells. *Biochem Biophys Res Commun* 1996, **226**(2), 329–334.
24. Nakajima-Iijima S, Hamada H, Reddy P, Kakunaga T. Molecular structure of the human cytoplasmic β-actin gene: interspecies homology of sequences in the introns. *Proc Natl Acad Sci USA* 1985, **82**, 6133–6137.
25. Yee C, Krishnan-Hewlett I, Baker CC, Schlegel R, Howley PM. Presence and expression of human papillomavirus sequences in human cervical carcinoma cell lines. *Am J Pathol* 1985, **119**, 361–366.
26. Pater MM, Pater A. Human papillomavirus types 16 and 18 sequences in carcinoma cell lines of the cervix. *Virology* 1985, **145**, 313–318.
27. Hiyama E, Hiyama K, Ishioka S, *et al.* Telomerase activity in small-cell and non-small-cell lung cancers. *J Natl Cancer Inst* 1995, **87**, 895–902.

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