

• BASIC RESEARCH •

Telomere and telomerase in the initial stage of immortalization of esophageal epithelial cell

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Abstract

AIM: To search for the biomarker of cellular immortalization, the telomere length, telomerase activity and its subunits in cultured epithelial cells of human fetal esophagus in the process of immortalization.

METHODS: The transgenic cell line of human fetal esophageal epithelium (SHEE) was established with E₆E₇ genes of human papillomavirus (HPV) type 18 in our laboratory. Morphological phenotype of cultured SHEE cells from the 6th to 30th passages, was examined by phase contrast microscopy, the telomere length was assayed by Southern blot method, and the activity of telomerase was analyzed by telomeric repeat amplification protocol (TRAP). Expressions of subunits of telomerase, hTR and hTERT, were assessed by RT-PCR. DNA content in cell cycle was detected by flow cytometry. The cell apoptosis was examined by electron microscopy (EM) and TUNEL label.

RESULTS: SHEE cells from the 6th to 10th passages showed cellular proliferation with a good differentiation. From the 12th to the 16th passages, many senescent and apoptotic cells appeared, and the telomere length sharply shortened from 23kb to 17kb without expression of hTERT and telomerase activity. At the 20th passage, SHEE cells overcame the senescence and apoptosis and restored their proliferative activity with expression of telomerase and hTERT at low levels, but the telomere length shortened continuously to the lowest of 3kb. After the 30th passage cells proliferation was restored by increment of cells at S and G2M phase in the cell cycle and telomerase activity expressed at high levels and with maintenance of telomere length.

CONCLUSION: At the early stage of SHEE cells, telomeres are shortened without expression of telomerase and hTERT causing cellular senescence and cell death. From the 20th to the 30th passages, the activation of telomerase and maintenance of telomere length show a progressive process for immortalization of esophageal epithelial cells. The expression of telomerase may constitute a biomarker for detection of immortalization of cells.

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INTRODUCTION

Telomerase activity was demonstrated in cancer of digestive tract^[1-3], such as gastric^[4-7], hepatic^[8-10] colorectal^[11-13] and esophageal cancers^[14-19]. Inhibition of telomerase activity will be a new therapeutic for cancer^[20-26]. Telomerase activity can be used as a diagnostic marker for cancer^[27-30]. Normal mammalian cells grow in cultural medium with a limited number of passages before entering senescence and death^[31], which are associated with shortening of telomere. Telomeres are specialized structures at chromosomal ends that are composed of TTAGGG DNA repeats^[32]. Telomerase is a ribonuclear protein complex, which contains human telomerase reverse transcriptase (hTERT) as a catalytic domain, and human telomerase RNA component (hTR)^[33]. Telomeres cap chromosomal ends perform the function of preventing abnormal chromosomal fusions and rearrangement^[34]. However, each time a cell divides, the most distal part of the chromosome is incompletely duplicated and the telomere becomes shorter. Critically short telomeres enable the formation of aberrant chromosomal structures resulting in growth arrest or senescence^[35]. With expression of telomerase or hTR and hTERT, the length of telomere extends to maintain the life span of the cells. There are other roles of telomerase in immortal and malignant lesion, such as proliferative potential^[36], delaying senescence^[37, 38], promoting cell cycle, cell immortalization and carcinogenesis^[39].

Recently, many papers have indicated that human papillomavirus (HPV) are the important etiological factor in esophageal carcinoma^[40-42]. Induced by HPV 18 E₆E₇ genes, we established an immortalized cell line (SHEE) from the esophageal epithelium which underwent^[43-46] malignant transformation^[47, 48]. Changes of telomere length and telomerase activity in the cell line are not clear at this early stage, nor is which criteria to use to detect immortalization of cells and what the relationship between telomerase and cell phenotype in SHEE cells is. The goal of this study is to explore when telomerase activity appears in the immortalized progressive process and study the relationship between telomerase and cellular phenotype.

MATERIALS AND METHODS

Cell culture and EM examination

The SHEE cell line was a kind of immortal embryonic esophageal epithelium induced by E₆E₇ genes of human papillomavirus (HPV) type 18 in our laboratory^[43]. The continual growth cells from the 6th to 30th passages were routinely cultivated in flasks and the 24-well plates (Corning Co.) with culture medium 199 (Gibco), 100mL·L⁻¹ bovine serum, 100u penicillin and streptomycin in a humidified atmosphere of 50mL·L⁻¹ CO₂ and 950mL·L⁻¹ air. The cell shape and size, anchorage-dependent growth and contact-inhibited growth were examined by phase contrast microscopy. For electron microscopic assessment, cells were spun to form a pellet and fixed with 25g·L⁻¹ glutaraldehyde. They were dehydrated in graded ethanol and

embedded in Araldite. Ultra-thin sections were cut with glass knives and mounted on copper grids. They were contrasted for 15 min with uranyl acetate and for 3 min with lead citrate. The sections were examined by electron microscope (Hitachi, H-300).

Cell proliferative Cycle and apoptosis

Cells cultured in the flask were digested, washed twice with PBS, fixed by 70% alcohol, prepared as single-cell suspension and stored at 4°C. Cells were stained with propidium iodide (Sigma) and analyzed with flow cytometry (FACSsort, B-D Co.). The percentage of cells in various stages of the cell cycle, the apoptotic cell rate (AI) and proliferation index ($PI = S + G_2M / G_0G_1 + S + G_2M$) were calculated. These cells on the glass coverslips within the 24-well plate were incubated with $10 \text{ mg} \cdot \text{L}^{-1}$ proteinase K for 15 min at room temperature. After the quenching of endogenous peroxidase, labeled nuclei with TUNEL (*In-Situ* Death Detection kit, Boehringer Mannheim Co.) were detected according to the instructions of the manufacturer. The brownish nucleus was considered positive apoptotic nucleus.

Telomere length analysis^[49]

The genomic DNA of 10^6 - 10^8 cells was extracted. The telomeric restriction fragment (TRF) was measured by Southern blot. Briefly, $20 \mu\text{g}$ of genomic DNA was digested with *Hinf*I and run on $7 \text{ g} \cdot \text{L}^{-1}$ agarose gel with marker DNA/*Hind* III. After electrophoresis the gel was blotted to nylon membrane (HybondTM N⁺, Amersham, Life Science) and hybridized to the Dig-labeled probes (CCCTAA)³ at 50°C in $5 \times \text{SSC}$, $1 \text{ g} \cdot \text{L}^{-1}$ Sod. n-Lauroylsarcosine (SLS), $0.2 \text{ g} \cdot \text{L}^{-1}$ SDS for 12-16h and washed twice at room temperature in $2 \times \text{SSC}$, $1 \text{ g} \cdot \text{L}^{-1}$ SDS for 5min, once at 50°C in $1 \times \text{SSC}$, $1 \text{ g} \cdot \text{L}^{-1}$ SDS for 10min, twice at 50°C in $1 \times \text{SSC}$, $1 \text{ g} \cdot \text{L}^{-1}$ SDS for 10min, twice at 50°C in $0.1 \times \text{SSC}$, $1 \text{ g} \cdot \text{L}^{-1}$ SDS for 5min, stained with NBT/BCIP and the median points were measured to obtain the mean telomere length^[50].

Telomerase activity assay

Telomerase activity was measured using the telomeric repeat amplification protocol (TRAP). Frozen samples were homogenized in 10 - $50 \mu\text{L}$ of ice-cold lyses buffer ($10 \text{ mmol} \cdot \text{L}^{-1}$ Tris-HCl, pH7.5, $1 \text{ mmol} \cdot \text{L}^{-1}$ EGTA, $0.1 \text{ mmol} \cdot \text{L}^{-1}$ Benzamidine, $5 \text{ mmol} \cdot \text{L}^{-1}$ β -mercaptoethanol, $5 \text{ g} \cdot \text{L}^{-1}$ CHAPS, $100 \text{ mL} \cdot \text{L}^{-1}$ glycerol). After 30min of incubation on ice, the lysate was centrifuged at 12000 g for 20min at 4°C. TRAP-eze Telomerase Detection Kit (Oncor Inc.) reaction was performed using $1 \mu\text{L}$ lysate or $1/10$ diluted lysate, $2.5 \mu\text{L}$ $10 \times \text{TRAP}$ buffer ($200 \text{ mmol} \cdot \text{L}^{-1}$ Tris-HCl, pH8.3, $15 \text{ mmol} \cdot \text{L}^{-1}$ MgCl_2 , $630 \text{ mmol} \cdot \text{L}^{-1}$ KCl, 0.5% Tween 20, $10 \text{ mmol} \cdot \text{L}^{-1}$ EGTA, $1 \text{ g} \cdot \text{L}^{-1}$ BSA), $0.5 \mu\text{L}$ $2.5 \text{ mmol} \cdot \text{L}^{-1}$ dNTP, $0.5 \mu\text{L}$ Ts primer, $0.5 \mu\text{L}$ TRAP primer mix, $19.5 \mu\text{L}$ water, $0.5 \mu\text{L}$ taq ($2 \times 10^6 \text{ u} \cdot \text{L}^{-1}$). After incubation at 30°C for 30min, the reaction mix was immediately transferred to 94°C and performed PCR (GeneAmp PCR System 2400, PE, USA) at 94°C for 30s, 55°C for 30s, for 30 cycles. PCR products were separated in a non-denaturing $120 \text{ g} \cdot \text{L}^{-1}$ PAGE in $1 \times \text{TBE}$ at $5 \text{ V} \cdot \text{cm}^{-1}$. The gel was stained using AgNO_3 and was photographed.

Subunits of telomerase analysis

The activities of telomerase were performed by hTERT and hTR analysis. Analysis of expression of hTR and hTERT was determined by reverse transcription-PCR (RT-PCR) amplification in contrast with house-keeping gene GAPDH. Total RNA was isolated from the cell using GstracTM RNA Isolation Kit (Maxim Biotech, Inc.). cDNA was synthesized from $10 \mu\text{g}$ of total RNA using Ready-to-use First Strand cDNA Synthesis Kit (Maxim Biotech, Inc.). PCR reaction was performed using $2 \mu\text{L}$ aliquots of the reverse-transcribed cDNA,

$2.5 \mu\text{L}$ $10 \times \text{PCR}$ buffer, $1.5 \mu\text{L}$ $25 \text{ mmol} \cdot \text{L}^{-1}$ MgCl_2 , $2.5 \mu\text{L}$ $1.0 \text{ mmol} \cdot \text{L}^{-1}$ dNTP, $2.0 \mu\text{L}$ specific primers, $14 \mu\text{L}$ water, $0.5 \mu\text{L}$ taq ($2 \times 10^6 \text{ u} \cdot \text{L}^{-1}$). hTERT mRNA was amplified using the primer pair: 5'-CGGAAGAGTGCTCTG-GAGCAA-3' and 5'-GGATGAAGCGAGTCTGGA-3' for 31 cycles (94°C for 45s, 55°C for 45s, 72°C for 90s). hTR was amplified using the primer pair: 5'-TCTAACCCTAACTGAGAAGGGCGTAG-3' and 5'-GTTTGCTCTAGAATGAACGGTGGGAAG-3'. GAPDH was amplified using the primer pair: 5'-GAAGGTGAAGGTCCGAGTC-3' and 5'-GAAGATGGTGATGGGATTTC-3' for 33 cycles (94°C for 60s, 55°C for 60s, 72°C for 60s). PCR products of each sample were subjected to electrophoresis in a $15 \text{ g} \cdot \text{L}^{-1}$ agarose gel containing $0.5 \text{ mg} \cdot \text{L}^{-1}$ ethidium bromide.

RESULTS

In the initiated passages, the cells in the 6th to the 10th passages were uniform in size and shape (Figure 1A), and grew as an even monolayer with characteristics of anchorage-dependent and attachment-inhibited growth. Cells continuously cultured in the 12th to the 16th passages exhibited morphologic changes in which cells were enlarged and flattened and exhibited differentiation and senescence. When many cells had shrunk, were round and floated freely, the majority underwent apoptosis and cell death with a few cells surviving (Figure 1B). Over coming senescence and apoptosis, the cells of the 20th passage restored their proliferation capacity (Figure 1C). After 30 passage the cells proliferated again and exhibited diphasic differentiation, a portion of cells displayed the undifferentiated basal epithelium and the other portion displayed differentiated squamous epithelium (Figure 1D).

Proliferative index (PI) and apoptosis

The PI of cell in 10, 16, 20, 30 the passage were 25.5%, 17.3%, 43.3%, and 43.0% respectively (Figure 2, A, B, C, D). Apoptotic cells index (AI) was in 10th passage, 7.3%; 16th, 57.5%; 20th, 5.7% and 30th, 7.5%. The cells in the 16th passage were at the stage of senescence and death, and the 10th, 20th and 30th were at their proliferated stages at various levels. Cells in G_0G_1 phase were identified as the differentiated cells containing some senescent cells.

The TUNEL assay was also used to characterize the biological features of cells apoptosis. Many TUNEL-positive nuclei were observed in cells of passage 16th (Figure 3), and in a few cells in other passages. By EM examination, many apoptotic cells revealed rounded and shrunken nuclei with condensed chromatin stuck closely to the nuclear membrane (Figure 4).

Telomere length

Following continuous growth of the cells, the telomere length of the cells in the 6th to 10th passages exhibited shortening from a mean size of 23kb in the normal esophageal mucosa to 17kb at passage 10. At 20th passage telomere length was shortened continually to the shortest 3kb, but maintained till the 30th passage (Figure 5).

Telomerase activity, expression of hTR and hTERT

Expression of hTR and hTERT in SHEE cells was determined by RT-PCR. The hTERT expression was positive in the 20th and 30th passages, but negative in the 10th passage (Figure 6A). Cells of the 10th, 20th and 30th passage showed positive expression of hTR (Figure 6B). House-keeping protein GAPDH was used as a control (Figure 6C). Comparing the variation of telomerase activity in SHEE from the 10th to the 30th passage, cells of the 10th passage were negative, while the 20th was weak and the 30th was apparent. The positive control of human esophageal squamous cell carcinoma expressed the highest telomerase activity (Figure 7).

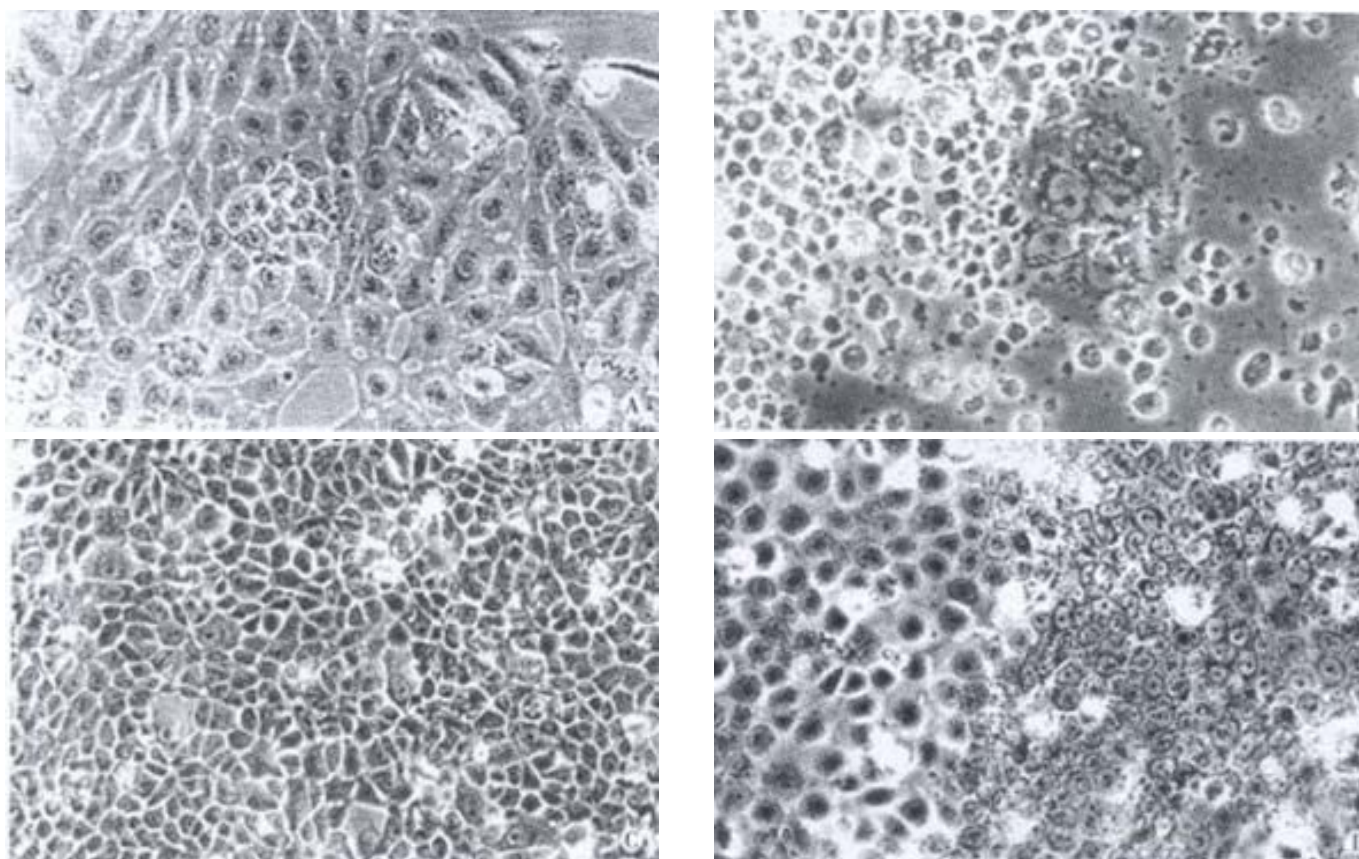


Figure 1 Morphologic changes in living cells of SHEE.

A: Cell in 6-10th passages showed differential phenotype (phase-contrast microscopy, Ph $\times 400$);

B: Cells of 16th passage displayed apoptosis with a few of cells survived (Ph $\times 400$);

C: Cells of 20th passage displayed hyperplasia (Ph $\times 200$);

D: Cells of 30th passage displayed proliferative activity with diphasic differentiation (Ph $\times 400$).

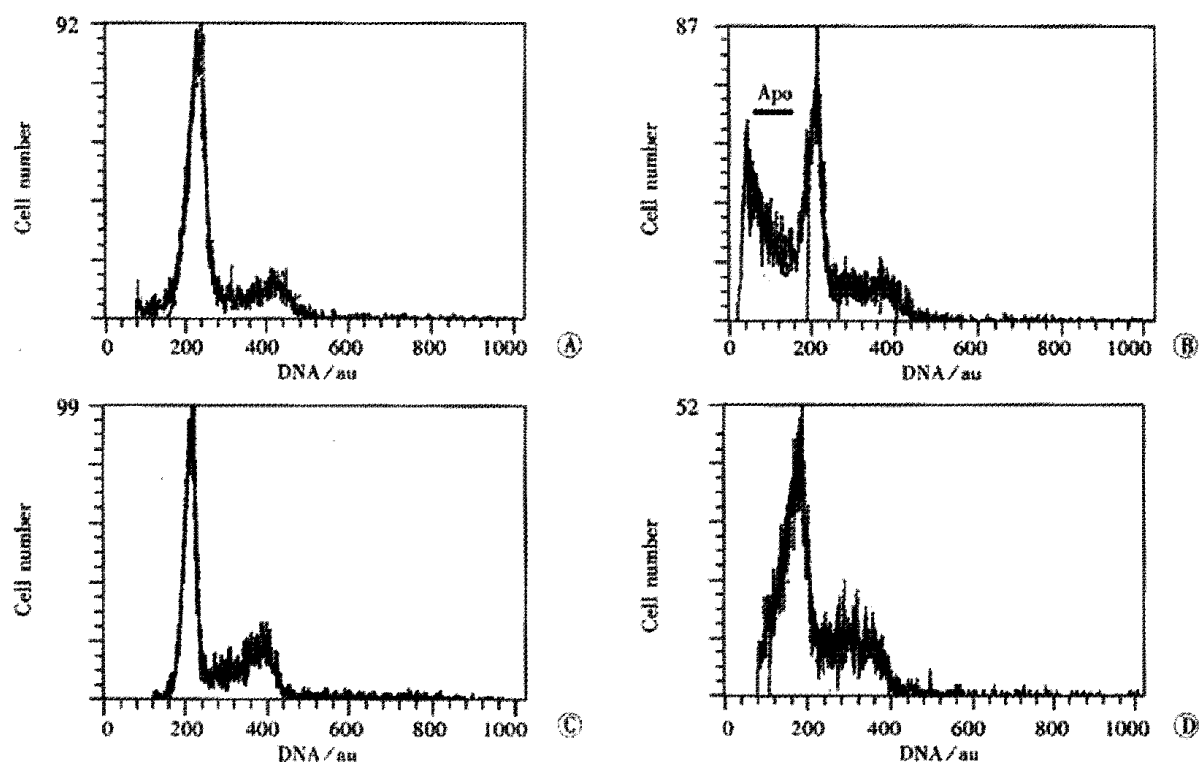


Figure 2 DNA histogram of SHEE cells.

A: 10th passage; B: 16th passage; C: 20th passage; D: 30th passage.

Apo: Apoptotic peak; AU: Arbitrary unite.



Figure 3 TUNEL positive apoptotic nuclei in 16th passage of SHEE. (x400)

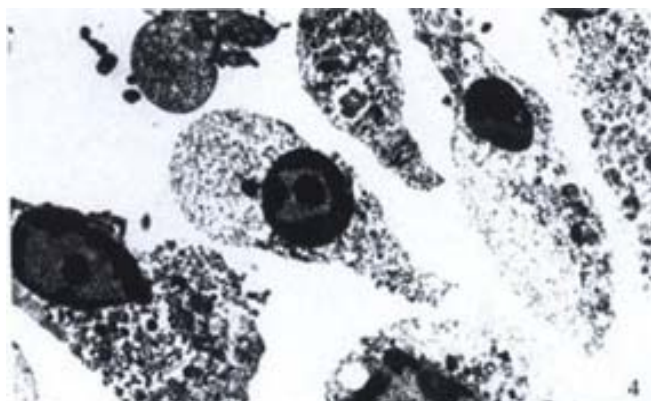


Figure 4 Electron-photomicrograph of apoptotic cells in 16th passage. Shrunken cells and rounded nuclei with margined condensed chromatin. (EM x3000)

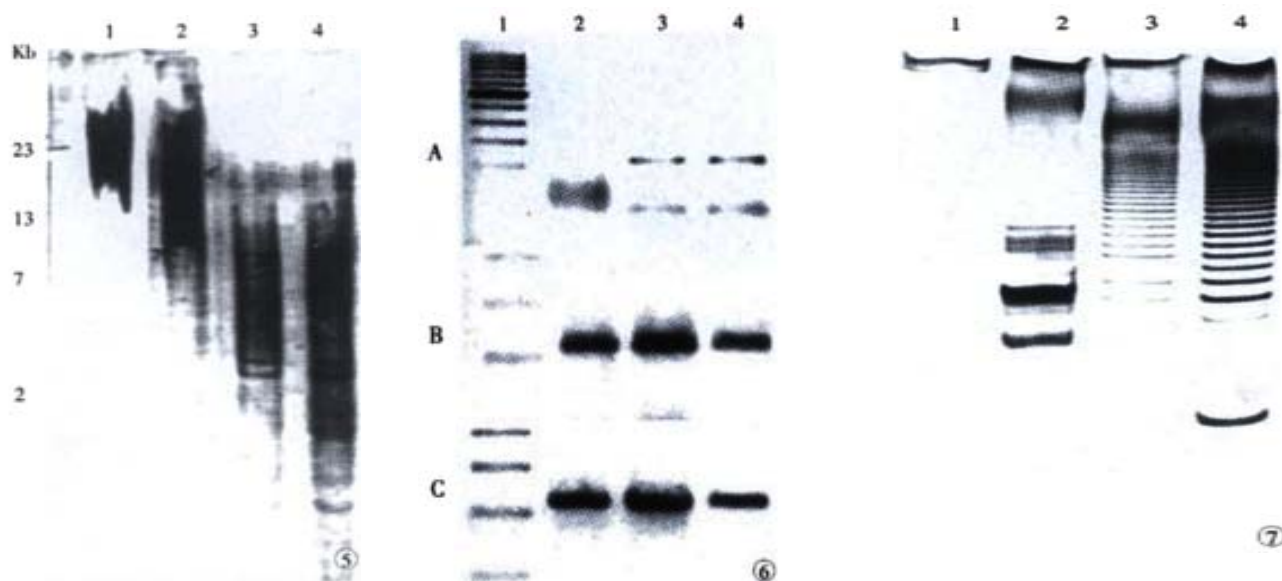


Figure 5 Telomere length of SHEE series using Southern blot.

1: Normal esophagus; 2: Cells of 10th passage; 3: Cells of 20th passage; 4: Cells of 30th passage.

Figure 6 Gel electrophoretogram of hTERT(A), hTR (B) and GAPDH (C).

Lane 1: marker; Lane 2: 10th passage; Lane 3: 20th passage; Lane 4: 30th passage.

Figure 7 Measurements of telomerase activity using TRAP assay.

Lane 1: 10th passage, negative; Lane 2: 20th passage, weak; Lane 3: 30th passage, positive; Lane 4: human esophageal squamous cell carcinoma, strong positive.

The relationship between telomerase activity and cellular proliferation

At an early stage in the immortalization process, SHEE cells could be divided into three stages. At the primary stage, telomerase activity and expression of hTERT was absent and the telomere length shortened. The cells of SHEE were proliferated and differentiated and then they were senescent and apoptotic cells in the culture. At the early immortal stage, cells exhibited telomerase activity and its subunits hTERT at low levels with telomere length being continually shortened and proliferation restored. At the immortal stage, telomerase and hTERT were expressed in high level and telomere length maintained, accompanied by cell proliferation. From 6th to 30th passages the cells expressed hTR.

DISCUSSION

After transferring the HPV18E₆E₇ genes to the fetal esophageal

epithelial cells, we established an immortal cell line designated SHEE. In order to focus on the process of immortalization, we monitored the dynamic changes of telomere length and telomerase activity of SHEE cells for extended periods of time (from the 6th passage to the 30th passage). At the primary stage the cells of passages 6-10 appeared as differentiated squamous epithelial cells with telomere length shortened without telomerase expression, after which the cells of passages 12-16 became senescent and underwent cell death. After overcoming the senescence and cell death, cells of the 20th passage expressed telomerase activity at low levels, where the length of shortened telomere was not maintained, but the proliferation of cells was restored. At the 30th passage, the cells exhibited a higher level of telomerase activity, hTR and hTERT, with maintained telomere length and continual proliferation of cells (immortalization). Therefore, one can suggest that telomerase activity and maintenance of telomere length might be necessary for immortalization of human esophageal epithelium *in vitro*.

To investigate the immortalization process, we used the SHEE cell model to define steps in aging of cells. After transduction of E₆E₇ genes from HPV type 18 normal cultured cells proliferate until they reach a discrete point (passage 12 times) in which the population growth ceases and develops to senescence. This period is termed the M1 stage of aging^[51]. After M1, a large amount of cells dead to reach a “crisis” point with a few cells survival. This period is termed the M2 stage of aging^[52]. Both M1 and M2 are therefore potential suppression pathways for tumorigenesis. The telomerase activity is sufficient to allow the cultured cells to escape from crisis^[53]. In our experiment cells transfected with HPV, cell proliferated for an extended period of time, after that cells encountered senescence (M1) and apoptosis (M2). Overcome the M1 and M2, cells exhibited accelerant hyperplasia with telomerase activity.

Recent evidence suggests that viral oncogenes might directly up-regulate telomerase activity^[54], such as HCV core protein, EBV-, HPV- and SV40 T antigen-expressing cell clones^[55-58]. In our data the increase of hTR in the primary cells of SHEE might be directly or indirectly affected by the HPV viral oncogenes. Previous reports also have shown that telomerase activity can be achieved by the E₆ and E₇ protein of HPV^[59]. HPV 16 E6 oncoprotein was capable of inducing telomerase activity in monolayer cultures of proliferating keratinocyte^[60]. It was more likely that HPV E6/E7 transcription or other additional alterations, as chromosome instability^[61,62] was prerequisite for induction of telomerase activity in proliferating cells. This hypothesis fits very well with our data on HPV-mediated immortalization of cells *in vitro*.

In summary, immortal cell line of the SHEE, up to 30th passage, may be divided into three stages. At the primary stage, telomerase activity and hTERT of cells were absent but hTR is positive with telomere length shortening and the cells became senescent and apoptotic. At the early-immortalized stage, the telomerase activity and hTERT expressed at low level and telomere length shortened continuously, but underwent cell hyperplasia occurred. At the immortalized stage, the telomerase activity and its two subunits expressed at a high level, telomere length was maintained and cell proliferation continued, in which the cells reached the stage of immortalization. The shortened telomere length and the activated telomerase activity display in a dynamic process. Our results prove that shortening of telomeres and absence of telomerase activity contribute to cellular senescence and cell death, and the activation of telomerase to maintain telomere length is necessary for immortalization. Therefore, the telomerase activity is the biomarker of immortalization of cell.

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