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• ESOPHAGEAL CANCER •

Progressive transformation of immortalized esophageal epithelial cells

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Abstract

AIM: To investigate the progressive transformation of immortal cells of human fetal esophageal epithelium induced by human papillomavirus, and to examine biological criteria of sequential passage of cells, including cellular phenotype, proliferative rate, telomerase, chromosome and tumorigenicity.

METHODS: The SHEE cell series consisted of immortalized embryonic esophageal epithelium which was in malignant transformation when cultivated over sixty passages without co-carcinogens. Cells of the 10th, 31st, 60th and 85th passages were present in progressive development after being transfected with HPV. Cells were cultivated in a culture flask and 24-hole cultural plates. Progressive changes of morphology, cell growth, contact-inhibition, and anchorage-dependent growth characteristics were examined by phase contrast microscopy. The cell proliferation rate was assayed by flow cytometry. The modal number of chromosomes was analyzed. HPV18E_6E_7 was detected by Western blot methods and activities of telomerase were analyzed by TRAP. Tumorigenicity of cells was detected with soft agar plates cultivated and with tumor formation in SCID mice.

RESULTS: In morphological examination the 10th passage cells were in good differentiation, the 60th and 85th passages cells were in relatively poor differentiation, and the 31st passage cells had two distinct differentiations. The characteristics of the 85th and 60th passage cells were weakened at contact-inhibition and anchorage-dependent growth. Karyotypes of four stages of cells belonged to hyperdiploid or hypotriploid, and bimodal distribution of chromosomes appeared in the 31st and 60th passage cells. All of these characteristics combined with a increasing trend. The activities of telomerase were expressed in the latter three passages. Four fourths of SCID mice in the 85th passage cells and one fourth of SCID mice in the 60th passage cells developed tumors, but the cells in the 10th and 31st passage displayed no tumor formation.

CONCLUSION: In continual cultivation of fetal esophageal epithelial cells with transduction of HPV18E_6E_7, cells from the 10th to the 85th passage were changed gradually from preimmortal, immortal, precancerous to malignantly transformed stages. All of these changes were in a dynamic progressive process. The establishment of a continuous line of esophageal epithelium may provide a *in vitro* model of carcinogenesis induced by HPV.

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INTRODUCTION

The cell line SHEE was derived from immortalized embryonic esophageal epithelium induced by gene E6E7 of HPV 18 in our laboratory[1,2] being cultivated and propagated over 100 passages. The 31st generation (SHEE31) had begun to express partial cell differentiation into two directions with some nests of cells with good differentiation and some with poor differentiation[3]. The 61st generation cells (SHEE61) were premalignant cells[4], and displayed a fully malignant transformation with a strong invasive potency at the 85th passage (SHEE85)[5]. We believe that this established cell line (SHEE), continually affected by expression of HPV, would change its biological characters such as cell proliferation, differentiation, chromosome and telomerase, and that this might be controlled by cytogenesis (chromosomes) and molecular genetics (genes).

In general, the immortalized or transformed cells caused by carcinogens are always accompanied by chromosome abnormality and mutation of gene[6]. The chromosome's changes are manifested in structure and the number of chromosomes[7,8]. All of these changes appear in the procedure from quantitative to qualitative changes. The length of telomere in living cells was continually shortened after cellular mitosis[9]. Because the somatic cells have no or lower levels of telomerase activity, telomere would be shortened, so it limits the division and lifespan of cells[10,11]. Immortal or malignant cells manifest telomerase activities, which can maintain the telomere length[12,13], so they will be immortal. With exposure of the early passage of immortal cell line to viral oncogenes, HPV or SV40T, conversion of these telomerase from negative expression to high levels of telomerase activity resulted[14]. Telomerase would be present in benign lesions and activated during the late stage of carcinogenesis[15].

Changes occurred in SHEE cells from the 10th to the 85th passage, with emphasis on their phenotypes, cytogenetic changes, telomerase activity and tumorigenicity, were studied in this paper. Phenotype of cells included the morphological changes of proliferation and contact-inhibition growth, and the modal number of chromosomes and the tumorigenicity, especially soft-agar culture and tumor formation in severely combined immuno-deficient (SCID) mice.

MATERIALS AND METHODS

Cell culture

The SHEE cells were routinely cultivated in culture medium 199 (GIBCO) with 10 % bovine serum, 100 units of penicillin and streptomycin in a humidified atmosphere of 5 % CO_2. Selected generations at the 10th passage (SHEE10), 31st passage (SHEE31 60th passage (SHEE60) and 85th passage (SHEE85) were inoculated in culture flask and 24-hole culture plate with glass slide inside.

Living cell examination

The cell shape, anchorage dependent and contact-inhibited growth were examined by phase-contrast microscopy.

Cell cycle analysis

Cultured cells of each passage were collected from suspended and digested cells, fixed by 70 % alcohol, then filtered through nylon mesh, to generate single-cell suspension (1×10^6/ml). The cells were stained with propidium iodide (Sigma) and were analyzed using flow cytometery (FCM) (FACSort Becton-Dickinson). Data of DNA of cells were collected and analyzed with Lysis II software, then a histogram was drawn and the cell percentages of each proliferation stage in the cell cycle and the number of cells more than 4n of DNA were calculated. The proliferation index formula: $S+G_2M/G_0G_1+S+G_2M$ and the cell amount at $preG_0G_1$ stage, the apoptotic cells, were calculated.

Cytogenetic analysis

Metaphase spreads were obtained using standard cytogenetic methods. Briefly, the culturing cells of each passage were preserved at 3-4 ℃ for 3-4 h to make cells on synchronous stage, then cultivated at 37 ℃ for 3-4 h, and added in 0.05 ug/ml colchicine for 1 h. Harvesting was by standard method and stained with Giemsa, 50-100 metaphases were scored for each line.

Telomerase activity assay[16]

Telomerase activity was measured using the telomeric repeat amplification protocol (TRAP). Frozen samples were homogenized in 10-50 μL of ice-cold lyses buffer (10 mmol· L^{-1} Tris-HCl, pH7.5, 1 mmol· L^{-1} EGTA, 0.1 mmol· L^{-1} Benzamidine, 5 mmol· L^{-1} βmercaptothanol, 5 g· L^{-1} CHAPS, 100 mL· L^{-1} glycerol). After 30 min of incubation on ice, the lysate was centrifuged at 12 000 g for 20 min at 4 ℃. TRAP-eze Telomerase Detection Kit (Oncor Inc.) reaction was performed using 1 μL lysate or 1/10 diluted lysate, 2.5 μL 10×TRAP buffer (200 mmol· L^{-1} Tris-HCl, pH8.3, 15 mmol· L^{-1} $MgCl_2$, 630 mmol· L^{-1} KCl, 0.5 % Tween 20, 10 mmol· L^{-1} EGTA, 1 g· L^{-1} BSA), 0.5 μl 2.5 mmol· L^{-1} dNTP, 0.5 μL Ts primer, 0.5 μL TRAP primer mix, 19.5 μL water, 0.5 μL taq (2 μ· L^{-1}). After incubation at 30 ℃ for 30 min, the reaction mixture was immediately transferred to 94 ℃ and performed PCR (GenAmp PCR System 2400, PE, USA) at 94 ℃ for 30s, 55 ℃ for 30s, for 35 cycles. PCR products were separated in a non-denaturing 125 g· L^{-1} PAGE in 1×TBE at 5V· cm^{-1}. The gel was stained using $AgNO_3$ and was photographed.

Soft agar assays

Four passages of cells (1×10^4) were cultivated in each hole of the 6-hole plastic plate (Corning Co.) which was covered with two layers agarose (Agarose, V312A, Promega), the bottom, 1 % and the upper. 0.5 %. The cells were incubated in 5 % CO_2 at 37 ℃ incubator for 40 d and the cell colony formations were scored every ten days. Each soft-agar cloning experiment was carried out at least in duplicate.

Tumorigenicity assays

In vivo tumor graft experiments were performed on the severely combined immunodeficient (SCID) mice (C.B-17/IcrJ-scid, Animal Lab of Chinese Academy of Medical Sciences). Cells of each passage were injected into the subaxillary skin of four mice with 1×10^6 cells for every one. Mice were observed weekly for two months and the tumor tissues were examined histopathologically.

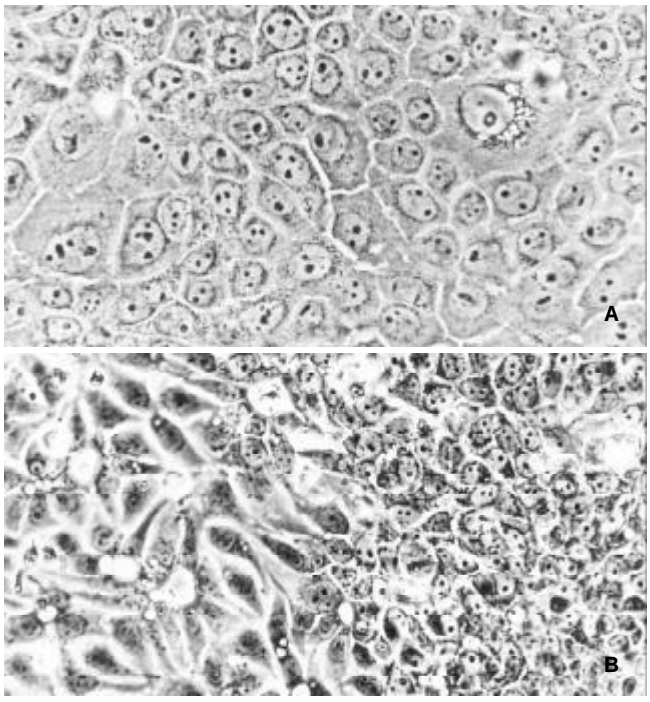
HPV18E_6E_7 assays

The protein expression of HPV18E_6E_7 was detected by Western blot method. The cells were washed three times with ice-cold PBS, then were lysed in buffer [50mM Tris-HC1, pH8.0, 150mM NaCl, 100 μg/ml phenyl-methyl-sulfonyl fluoride (PMSF), 1 % TritonX-100] for 30 min at ice. After removal of cell debris by centrifugation (12 000 g, 5 min), the protein concentration of lysates was measured by Bradford method. 50 μg proteins of different passage boiled for 5 min in sample buffer were separated by 10 % SDS-PAGE, transferred onto nitrocellulose membrane (Bio-Rad). Nonspecific reactivity was blocked by incubation overnight at 4 ℃ in buffer (10mM Tris-HCl, pH7.5, 150mM NaCl, 2 % Tween-20, 4 % bovine serum albumin). The membrane was then incubated with antibody of mouse anti HPV18E_6, (SC-264, Zhong Shan Biotech Co.), followed by reaction with anti-mouse IgG antibody. Reactive protein was detected by ECL chemiluminescence system (Amersham).

RESULTS

Proliferation and differentiation of SHEES

The cells of SHEE10 grew evenly on the flask. Cells appeared to have the characteristics of squamous epithelium (Figure 1, A) with multiangular outline and oval nucleus. SHEE31 were attached to the dish with partial differentiating into squamous epithelium and partial undifferentiated basal cells (Figure 1, B). The cells of SHEE60 and SHEE85 were differently shaped and sized and cells were crowded and overlapped (Figure 1C, D).



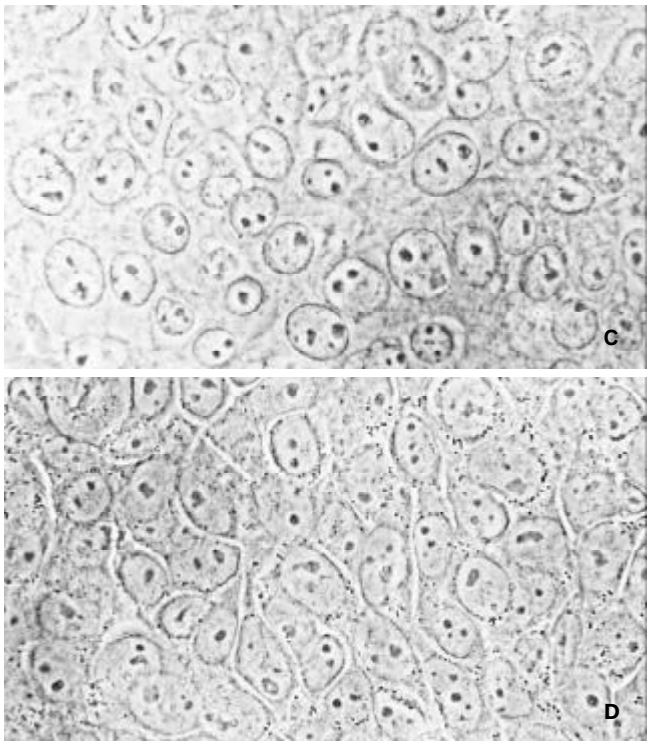


Figure 1 Morphology of SHEE cell (photographs of phase contrast microscope) A, SHEE10, good differentiation (×400); B, SHEE31, differentiated to two directions, well differentiated (left), and poorly differentiated (right) (×200); C, SHEE60, poor differentiation (×400); D, SHEE85 shows different shape and size with larger nucleolus (×400)

FCM analyzed cell cycle

In the DNA histogram (Figure 2), the distribution of DNA content of SHEE31 was similar to that of SHEE10, the proliferative indexes of SHEE10 and SHEE 31 were at the same level (32.0 %, 35.2 %), but different from SHEE60 (47.5 %) and SHEE85 (54.3 %). Of all DNA>4n cells there were SHEE10, 2.5 %; SHEE31, 4.7 %; SHEE60, 6.1 % and SHEE85, 7.2 %. This showed that heteroploid and hyperploid tumor cells increased with progressive culture of SHEE.

The modal number of chromosome

The number of chromosomes in SHEE10, SHEE31, SHEE60 and SHEE85 (Table 1) ranged between 32 and 196, and these chromosomes were mainly hyperdiploids and hypotriploids. Most cells of hypertriploids were found at SHEE60. Modal number of chromosomes at SHEE10 was 58-62, at SHEE31 and SHEE60 were bimodal distribution, 55-57, 61-63, and 58-60, 63-65 respectively, at SHEE85 was 59-65. The modal number from the 10th passage to the 85th passage increased slowly.

Table 1 Number of Chromosome in SHEE Series

Number of passage	Number of cell	≤46	47-57	58-68	≥69	modal number
SHEE10	91	17(18.7%)	45(49.5%)	22(27.7%)	4 (4.3%)	58-62
SHEE31	100	12(12.0%)	38(38.0%)	46(46.0)	4 (4.0%)	55-57,61-63
SHEE60	52	5 (9.6%)	13(25.0%)	22(42.3%)	12(23.1%)	58-60,63-65
SHEE85	85	4 (4.7%)	16(18.8%)	54(63.5%)	11 (12.9)	59~65

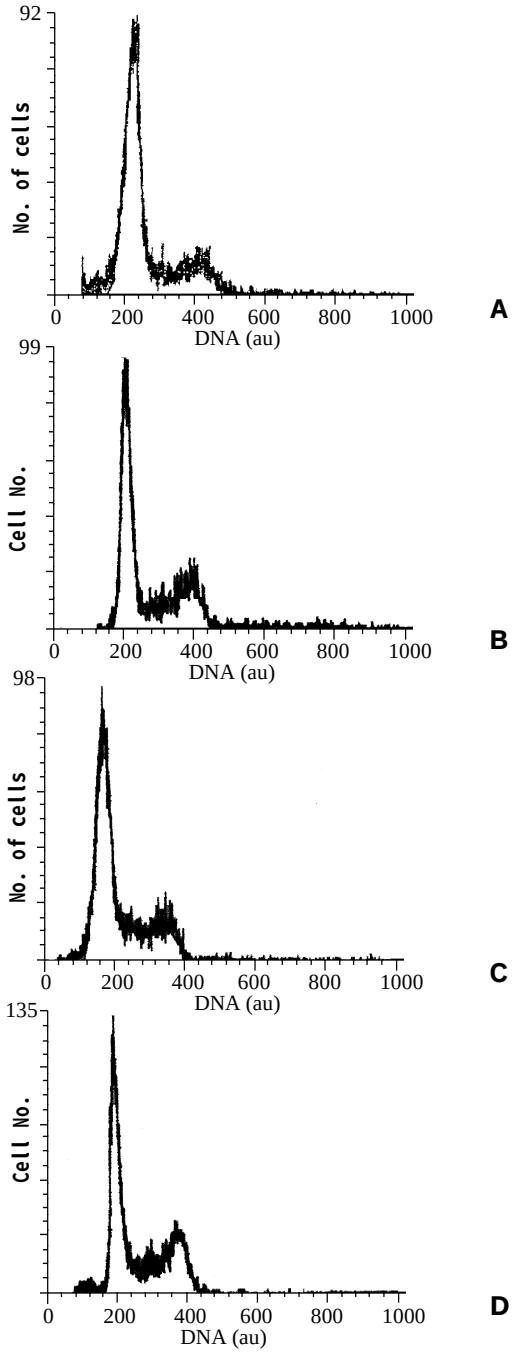


Figure 2 DNA histograms of SHEE. A, SHEE10; B, SHEE31; C, SHEE60; D, SHEE85; au, arbitrary unit.

Telomerase activity

Telomerase activation was absent in normal esophageal epithelium and SHEE10. The activity of telomerase appeared in the 31st passage, and it was strongly positive in SHEE60 and SHEE85.

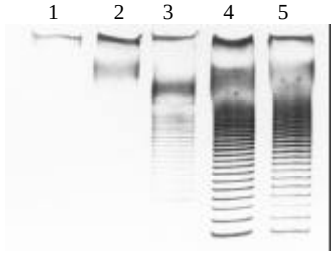


Figure 3 Activity of telomerase SHEE series 1, normal esophageal epithelium; 2, SHEE10; 3, SHEE31; 4, SHEE60; 5, SHEE85.

Expression of HPV18E$_6$E$_7$

The expression of HPV18E$_6$E$_7$ was examined by Western blot method. The figure (Figure 4) showed the expression of protein of HPV18E$_6$E$_7$ at cells of four stages of SHEE cell lines.

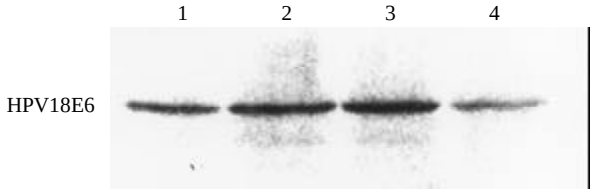


Figure 4 Western blot analysis of protein of HPV18E6 in SHEE 1, SHEE10; 2, SHEE31; 3, SHEE60; 4, SHEE85.

Tumorigenicity

SHEE10 could not grow on soft agar, but SHEE31, SHEE60 and SHEE85 could. SHEE31 formed small colonies, (less than 20 cells in a colony) compared to SHEE60 and SHEE85 in which large colonies (more than 50 cells in a colony) were formed. SCID mice inoculated with cells of SHEE10 and SHEE31 did not form tumors, but one quarter of SCID mice with inoculation of SHEE60 cells formed tumors with a latency period of over 2 months (data not shown). It was determined that a percentage of cells of SHEE60 manifested malignancy. All SCID mice inoculated with SHEE85 cells manifested tumor formation, which infiltrated into muscular layer histopathologically (Figure 5).

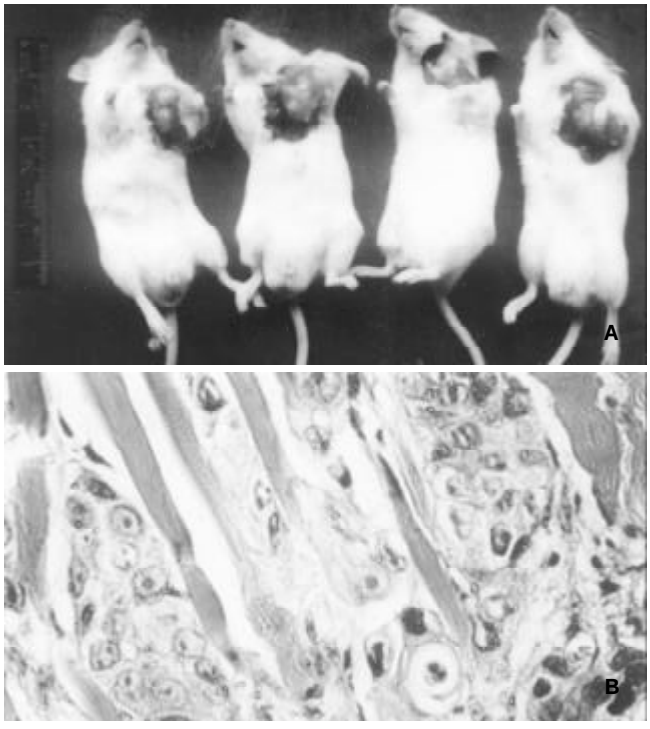


Figure 5 Heterotransplanting SHEE85 cells into SCID mice Tumors in right axiua (arrow) of SCID mice are found (A). Invasion of tumor cells is found in muscular layer (B). HE, ×400

DISCUSSION

Cellular proliferation, differentiation and apoptosis are fundamental life activities, and are also the growth markers of immortal cells. According to the DNA content and proliferation index, the cells of SHEE10, SHEE31, SHEE60 and SHEE85 all show proliferative characteristics. The proliferation index and cell numbers of polyploid (DNA>4n) of each passage were compared as a result of SHEE10<SHEE31<SHEE60 <SHEE85. SHEE31 cells showed differentiation in two directions: one displayed relatively large and multiangular cells with abundant cytoplasm and oval nucleus; the others displayed small cells with less cytoplasm and small round nucleus. SHEE60 cells overlapped to grow, which were differently shaped and poorly differentiated. SHEE85 cells were crowed in cultivation with cells of poor differentiation and received less contact-inhibition. Detecting anchorage growth and contact inhibition specificity by cultivation on the soft agar is of help to judge its malignant character[17,18]. The small colony formation of SHEE10 and SHEE31 cultivated in soft agar, and large colonies in SHEE85 and SHEE60 showed that the characteristics of anchorage-dependent growth decreased but tumorigenicity increased. A few tumors are formed in SCID mice incubated with SHEE60. So we judged that they were not at a fully malignant stage but at a premalignant stage. SHEE85 cells, which were transplanted into 4 SCID mice and developed tumors in all mice, expressed malignant transformation.

There were more hyperdiploid and hypotriploid in the chromosomes of four stages of SHEE cell lines. The separate modal number of chromosomes first appeared in SHEE31 and continued to SHEE60. The number of chromosomal sets and the percentage of hyperploid in SHEE31 varied between SHEE10 and SHEE60, and SHEE85 has more hypotriploid cells than the others. All above showed that chromosomes of SHEE series cell lines were unstable and more susceptible to malignant transformation by promoters[19, 20]. The changes of cytogenetics will control the proliferation and differentiation of cells[21].

In 1965, Hayflick reported that the culture life of human diploid fibroblast was limited to 50-100 generations, the same to epithelial cells. In 1985, Greider discovered the activity of telomerase. In 1994, Kim also identified a specific association between the telomerase activity and immortal cells or cancer cells. Telomerase activity was demonstrated in cancers of the digestive tract[22-24], such as gastric[25-29], hepatic[30-32] colorectal[33-35] and esophageal cancer[36-38]. The telomerase activity is possibly both a prerequisite and a diagnostic criterion for immortal cells[39]. Weitzman and Hahn believed T-antigen of SV40 and *ras* gene induce transformation of normal epithelium cells which require expression of telomerase activity[40,41], so over-expression of telomerase related to proliferation is the early event of cancer[42]. In our data the telomere was 30kb in length in the normal fetal esophageal cell, shortened to 17kb in SHEE 10, then further shortened to 3.5kb in SHEE 31, and then maintained at this level continually[43]. The telomerase, first appeared in the 20th passage, could not prevent shortening of telomere, because it was in a low-or noncatalytic function, therefore cells had difficulty to survive in cultivation before the 20th passage. The cells grew stably after the 31st passage. Our results indicated that immortalization of SHEE might require activation of telomerase.

Infection of HPV can cause karyotype confusion, such as breaking of chromosome, abnormal structure and number of chromosomes[44]. It also suggests that immortal esophageal cells induced by HPV18 E6E7 may affect the changes of chromosomes, and cause instability of genetic characters[45]. Viruses can cause loss of contact inhibition, decrease of adhesion between cells, confusion of cellular skeletal structure, and loss requiring to growth factor[46]. The virus genome inserts and integrates with the chromosome of host, causing the activation and expression of oncogenes[47]. HPV E6E7 protein is conjugated with anti-oncoprotein p53 and pRb[48], thus

causing loss of control of cellular growth, and encouraging the phenotyping production of cellular transformation. Previous reports have shown that activation of telomerase can be achieved by the E_6 and E_7 proteins of HPV[49]. It has been reported that E_6 could promote malignant change, while E_7 may cause benign neoplasm[50]. It has been indicated elsewhere that virogene HPV16 E6 alone can cause cellular malignant change[51]. In this experiment, we found that immortal cells contained HPV18 E6E7 and that, therefore, the SHEE series can gradually lead to the malignant transformation. It is also postulated that HPV is likelly be a major risk factor for esophageal cancer[52].

In summary, of all these cells, some underwent aging, apoptosis and died, whereas others proceeded to malignant transformation. To search the direction of development of cells, we evaluated these changes by referring to the cytogenetic index. In these four series of immortal cells, the chromosomes presented characteristics of hyperdiploid and hypotriploid along with separation of modal number. The positive activity of telomerase can help determine the cells that are progressing from preimmortal toward immortal stages. The SHEE60 passage showed initial partial malignant change which could be regarded as premalignancy. The cells of SHEE85 were in fully malignant transformation with tumor formation and invasive potential. In conclusion, it is possible to demonstrate multiple stages in the transformation process that are associated with different genetic and phenotypic characteristics.

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