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Immortalization of human extravillous cytotrophoblasts by human papilloma virus gene *E6E7*: sequential cytogenetic and molecular genetic characterization

Yuesheng Jin[a,b], Hui-chen Feng[c], Wen Deng[c], Hao Zhang[a], Mei Lv[a], Charlotte Jin[b], Sai Wah Tsao[c], Yok-Lam Kwong[a,*]

[a]*Departments of Medicine, University of Hong Kong, China*
[b]*Department of Clinical Genetics, Lund University Hospital, SE-221 85 Lund, Sweden*
[c]*Department of Anatomy, University of Hong Kong, China*

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Abstract Extravillous cytotrophoblast (EVCT) cultures from the normal placentas of three pregnant women were transfected by HPVE6E7. Sequential cytogenetic and molecular analyses were performed to delineate genetic events that may be critical for cell immortalization. One line, PE1-E6E7, was immortalized successfully, whereas 2 other lines, PE3-E6E7 and PE4-E6E7, could not be maintained beyond crisis. Before crisis, the majority of cells in all lines were karyotypically normal. During the early stages of crisis, there was progressive telomere shortening. Most cells were karyotypically abnormal, with extreme cytogenetic divergence and a predominance of telomeric association and dicentric chromosomes affecting many chromosomes. At the later stages of crisis, the karyotype became more convergent with a drastic decrease in nonclonal aberrations. In PE1-E6E7, after crisis the karyotype was complex, with frequent centromeric rearrangements in the form of isochromosomes and whole-arm translocations. There were unbalanced structural aberrations and numerical changes, including loss of chromosome 13, that could be traced throughout the evolution of the line. These findings support the concept that immortalization is a relatively rare and nonrandom event that occurs only in cells that have acquired the necessary or critical genetic alterations. Telomeric dysfunction may be an important mechanism leading to the acquisition of complex karyotypical aberrations.

1. Introduction

Immortalized normal cell lines have many potential experimental applications, including the analysis of molecular controls of specific gene expressions, identification of events during multistep neoplastic transformation, and the study of tumorigenesis mechanisms. We have developed an in vitro cell culture system for the immortalization of cells of different tissues by the transfection of the human papilloma virus (HPV) *E6E7* gene [1,2]. Although the HPVE6E7 is so far known to be involved only in the neoplastic transformation of cervical cells, these genes are useful tools for the immortalization of cells of other tissues.

Embryonic development requires a placenta that is supported by successful implantation of the trophoblasts, which are also termed extravillous cytotrophoblasts (EVCT) [3,4]. Abnormal differentiation and functional defects of EVCT may give rise to a variety of pregnancy-associated disorders, including spontaneous abortion, pre-eclampsia, and trophoblast diseases. The establishment of in vitro cell models of EVCT will be useful for defining the biologic characteristics and functional properties of these cells.

In this study, cell cultures from the normal placenta of three pregnant women were initiated and transfected by HPVE6E7 in an attempt to establish permanent EVCT cell lines. Furthermore, sequential cytogenetic and molecular cytogenetic analyses, including spectral karyotyping (SKY) and telomere fluorescence in situ hybridization (FISH), were performed at various points after HPV *E6/E7* transfection to delineate the cytogenetic patterns at different stages of cell immortalization as well as the chromosomal aberrations that might be critical for cell immortalization.

* Corresponding author. University Department of Medicine, Queen Mary Hospital, Division of Hematology/Oncology, Pokfulam Road, Professional Block, Hong Kong, China. Tel.: +852-28554597; fax: +852-2-974-1165.

E-mail address: ylkwong@hkucc.hku.hk (Y.-L. Kwong).

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2. Materials and methods

2.1. Initiation and immortalization of EVCT cells

The procedures for the initiation of primary cell culture and transfection of HPV *E6*/*E7* were as reported previously [5]. Briefly, fresh chorionic villous tissues from the first trimester normal placentas at the gestational age of 10 weeks from three individuals were obtained after informed consent. Primary cell cultures were transfected by a retroviral vector containing the HPV16 *E6*/*E7* genes (LSXN16E6E7, kind gift from Dr. D.A. Galloway, Fred Hutchison Cancer Research Center, Seattle, WA) as described [1]. At 3–6 months (passages 10–30), the lines PE1-E6E7, PE3-E6E7, and PE4-E6E7, deriving from each of the three women, entered a crisis stage where proliferation decreased progressively until the majority of cells stopped proliferating. At passage 27, PE3-E6E7 and PE4-E6E7 could not be maintained. However, a few cells from PE1-E6E7 overcame the crisis, grew actively, and became immortalized. They have since been subcultured for more than 100 population doublings (PD) without signs of senescence.

2.2. Cytogenetic analysis

Cytogenetic analysis of PE1-E6E7, PE3-E6E7, and PE4-E6E7 was performed at various points after E6E7 transfection (Table 1). For comparison, a previously established EVCT cell line B6 was also tested. Metaphase chromosomes were G-banded with Wright's stain and karyotyped according to the International System for Human Cytogenetic Nomenclature (1995) [6].

2.3. Telomere FISH

Telomere FISH was performed as described [7]. Quantification of individual telomere signals was performed by TEL-TELO software (kindly provided by Drs. P.M. Lansdorp and S. Poon, Mount Sinai Hospital, Toronto) [8,9].

2.4. SKY

SKY was performed with the probe kit SkyPaint mixture (Applied Spectral Imaging, Migdal Ha'Emek, Israel) according to the manufacturer's instructions. Image analysis and karyotyping were performed with the SkyVision Imaging System (Applied Spectral Imaging) equipped with a Zeiss Axioplan-2 fluorescence microscope (Carl Zeiss Jena GmbH, Jena, Germany).

3. Results

3.1. Characterization of PE1-E6E7

The full characterization of the line PE1-E6E7 has been described elsewhere (Feng HC, Choy MY, Deng W, Wong HL, Lau WM, Cheung ANY, Ngan HYS, Tsao SW. Establishment and characterization of a human first-trimester extravillous trophoblast cell line (cuhk-t1), submitted for publication). Essentially, it has retained the phenotype of normal EVCT, including the expression of cytokeratin 7, CD9, and HLA-G [4]. The potential of anchorage-independent growth on soft agar and tumorigenecity in nude mice were also tested. All results were negative.

3.2. PE1-E6E7 before crisis

At passage 1 (before crisis), the karyotype was normal, but nonclonal chromosomal changes were found in 13% (2/15) metaphase cells (Table 1). SKY at passage 4, corresponding to 22 PD, detected nonclonal chromosome aberrations in 2/30 metaphase cells. The frequency of nonclonal chromosomal changes reached 55% (16/29) metaphase cells at passage 8 (before crisis; Fig. 1A), and 38% of them were in the form of telomere association (TA) or dicentric chromosomes.

3.3. PE1-E6E7 during crisis

During crisis (passages 10, 17, and 20), telomeres were shortened progressively as shown by the telomere FISH (Fig. 1B). The majority of analyzed cell were karyotypically abnormal. Karyotypes were highly heterogeneous, and nearly half of the aberrations are TA or dicentric chromosomes (Fig. 2). Some cytogenetically unrelated clones emerged. The karyotypes became more convergent at passage 32, however, when three cytogenetically unrelated clones were identified and cells with nonclonal changes decreased drastically. Three independent cultures were cloned from passage 43 (i.e., PE1-C5P4, PE1-C4P4, and PE1-C3P4).

3.4. PE1-E6E7 after immortalization

When the three sublines PE1-C5P4, PE1-C4P4, and PE1-C3P4 were analyzed individually at passage 49, each was dominated by one clone. The clone PE1-C5P4 shared gain of chromosome 1 and loss of chromosome 13 with PE1-C3P4, whereas PE1-C4P4 did not share any aberration with the other two clones. At passage 53, a pooled culture was analyzed. Three clones that were cytogenetically unrelated but partly related to the clones found in P49 were identified. At passage 71 (more than 100 PD), all metaphase cells displayed relatively complex karyotypes with a chromosomal modal number within the hypotetraploid range (Fig. 3A). All of them shared karyotypic similarities, and three cytogenetically related subclones could be identified. Analysis of telomeres by FISH showed strong and even signals in all chromosomal ends (Fig. 3B). Clonal evolution, evidenced by cytogenetically related subclones, was evaluated. The inferred evolutionary pathways are illustrated in Fig. 4. Loss of chromosome 13, present in

Table 1
Cytogenetic profiles of the four EVCT cell lines by chromosome G-banding and spectral karyotyping

Lines/passage	Cells analyzed	Unrelated clones	No. of cells with nonclonal changes			Karyotypes
			Tas	Dic	Other	
PE1-E6E7						
P1 (pooled)	15	0	0	0	2	46,XY[13]
P4 (SKY)	30	0	0	1	1	46,XY[28]
P8	29	0	2	4	10	46,XY[13]
P10 (A)	59	2	8	1	29	45,X,dic(Y;1)(p11.3;p36.3)[7]/45,XY,tas(1;22)(p36.3;p13.3)[2]/ 46XY[13]
P10 (B)	16	0	5	0	9	46,XY[2]
P17	84	4	11	11	58	43~47,XY,add(1)(p36)[cp5]/43~44,idem,−13[2]/42~43, XY,dic(1;12)(p36;q24)[cp2]45~46,XY,del(4)(q11)[cp3] / 46,XY,del(12)(p11)[2]
P20	42	0	0	0	27	45~47,XY,add(1)(p36),−4,−13,+20[cp2]/46,XY[13]
P32	32	3	0	0	5	46,XY,i(12)(q10)[18]/45,XY,+der(1;16)(p10;p10),−7−16[5]/ 43,XY,+add(1)(q11),−7,−16[2]/46,XY[2]
P43 (SKY)	29		0	0	0	45,XY,+der(1;16)(p10;p10),del(7)(q11),−13,−16[16]/46,XY, der(1;2)(p2?;p2?),−13[7]/45~47,XY[6]
P49 C5/P4	25	0	0	0	0	47~49,XY,+1,+12,−13,add(16)(q11),+mar[cp25]
P49 C3/P4	32	0	0	0	0	76~78,X,de1(X)(q11),Y,+add(1)(p36),+3,+5,+6,der(7;11) (q10;q10),der(8;15)(q10;q10),+12,+12,−13,−13,+19, +20,+mar[cp32]
P49 C4/P4	23	0	0	0	1	46,XY,add(7)(q11),i(12)(q10)[cp22]
P53 (pooled)	53	3	0	0	0	50~51,XY,+1,+12,+20,+mar[cp12]/47~50,idem,add(1) (q11)[cp15]/50~51,idem,add(2)(p11)[cp25]/44~45,XY, +add(1)(q21),del(7)(q11),−13,−15,−16[cp8]/46,XY,i(12)(q10)[3]
P71 (SKY)	33	0	0	0	0	81,XXYY,−4,der(6;15)(q10;q10),der(7;8)(p10;q10),del(10) (q22)x2,del(11)(q11),−13,−13,−16,−17,der(19)ins(19;20) (q13;?)t(17;19)(?;q13),der(20)dup(20)(?)t(17;20)(?;?),−22 [7]/ 83,idem,der(1;5)(p10;q10)[21]/85,idem,der(1;20)(p10;q10), del(4)(q21),+11[5]
PE3-E6E7						
P10	27	7	0	0	6	46,XX,der(7)t(X;7)(q11;q36[4]/46,XX,dup(7)(q36q22)[3]/ 46,XX,der(7)t(7;11)(q36;q13)[3]/46,XX,add(7)(q36)[2]/ 46,XX,der(7)t(1;7)(q21;q36)[2]46,XX,der(7)t(7;8)(q36;q13)[2]/ 46,XX,dic(7;17)(q36;p11)[2]/46,XX[3]
P17	48	7	3	3	10	46,XX,dup(7)(q36q22)[14]/46,XX,der(7)t(1;7)(p32;q36)[3] 46,XX,der(7)t(3;7)(q21;q36)[3]/46,XX,der(7)t(X;7)(q11;q36) [2] / 46,XX,der(7)t(1;7)(q21;q36)[2]/46,XX,der(7)t(5;7)(q13;q36)[2]/ 46,XX,der(7)t(7;8)(q36;q13)[2]/46,XX[4]
P20	33	3	3	0	17	46,XX,add(7)(q36)[5]/46,XX,dup(7)(q32q36)[3]/del(4)(p11) [3]/ 46,XX[2]
PE4-E6E7						
P10	19	0	0	0	0	45,XY,−13[19]
P13	24	0	0	0	0	45,XY,−13[18]/45,idem,add(16)(q24)[6]
P17	41	0	0	0	0	45,XY,−13[28]/44,idem,−16[3]/45,idem,add(16)(q24)[10]
P20	26	0	0	0	0	45,XY,−13[18]/44,idem,−16[2]/45,idem,add(16)(q24)[6]
P26	23	0	0	0	0	45,XY,−13[17]/45,idem,add(16)(q24)[6]
B6						
P49	24	0	0	0	0	79~82,XXY,−Y,−2,add(2)(p25),−4,−6,−13,−13,−14, add(14)(q24),−15,−16,−17,−19,+i(21)(q10)x1~2 [cp9]/ 77~79,idem, add(9)(p13) [cp13]/78~81,idem,i(8)(q10) [cp2]

Abbreviations: Tas, telomere association; Dic, dicentric chromosome.

most of clones that survived crisis, was an apparently early event in those immortalized clones.

3.5. PE3-E6E7

This line was studied only during the crisis stage (at passages 10, 17, and 22). The majority of cells displayed chromosomal aberrations. Clonal chromosomal changes could be identified in all passages analyzed. The distal part of the long arm of chromosome 7 was involved in all clones.

3.6. PE4-E6E7

Cytogenetic analysis was performed only during the crisis (at passages 10, 13, 17, 10, and 26). All cells analyzed were karyotypically abnormal. In contrast to lines

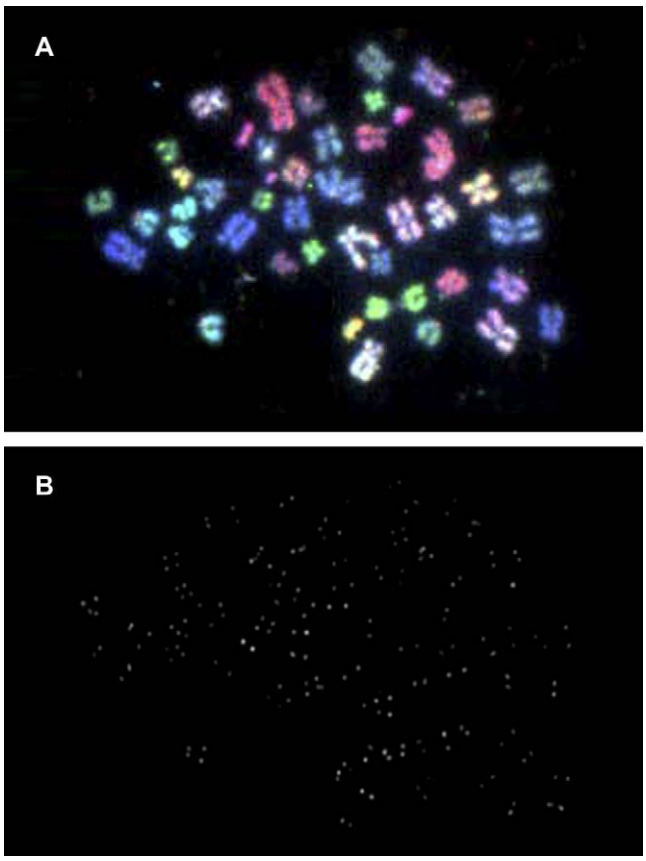

Fig. 1. PE1-E6E7 cells before crisis. (A) Spectral karyotyping of PE1-E6E7 cells. (B) Corresponding telomere fluorescence in situ hybridization showing weak signals on the majority of chromosomal ends.

PE1-E6E7 and PE3-E6E7, karyotypes in this line were homogeneous and stable. Loss of chromosome 13 was found in all metaphase cells analyzed, and in the majority of them, this was as the sole chromosomal anomaly. The clone with loss of chromosome 13 was present at different time points and dominated the karyotype (Fig. 5).

3.7. B6-E6E7

As a comparison, this immortalized line was also analyzed karyotypically. Fairly complex karyotypes with triploid modal chromosomal numbers were found. Numerical changes, including loss of chromosome 13, were predominant.

4. Discussion

We have described the karyotypic changes that take place during the immortalization of EVCT lines. In addition to being an in vitro model of EVCT, these lines have also provided insights into the early events of cellular immortalization. We showed that the majority of cells in pre-crisis, corresponding to the point before senescence (the first replicative arrest or mortality stage 1), were

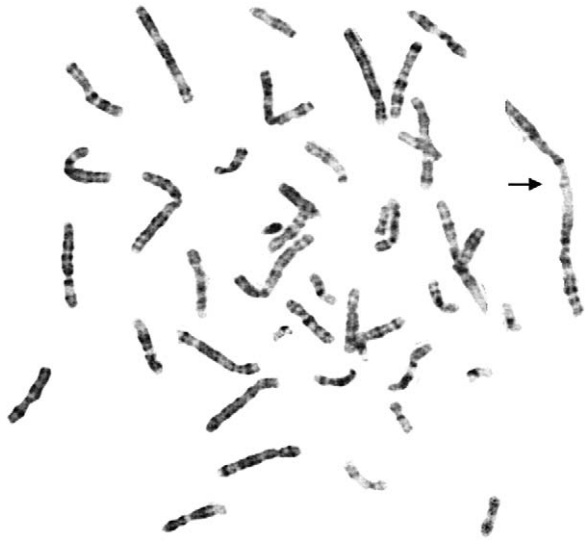

Fig. 2. A metaphase spread of PE1-E6E7 at passage 17. A dicentric chromosome dic(1;2) is indicated by an arrow.

karyotypically normal, with a low frequency of chromosomal aberrations. During the early stages of crisis, a progressive telomeric shortening occurred. At this time, most cells showed karyotypic changes and an extreme cytogenetic divergence. A characteristic cytogenetic feature during crisis was the predominance of TA or dicentric chromosomes affecting many chromosomes. In the later stages of crisis (the second replicative arrest or mortality stage 2), however, the karyotypic patterns tended to be more homogeneous. Finally, only a few clones eventually survived the crisis and became immortalized, with more complex kayotypes arising. These observations support the concept that immortalization is a relatively rare and nonrandom event in a given cellular population and occurs only in a few cells that have acquired the necessary or critical genetic alterations.

To define the genetic changes important for cell immortalization, karyotypic patterns before and after crisis were evaluated. Conceptually, the majority of chromosomal abnormalities during crisis are random events. Some chromosomal events, however, may confer growth advantage, thus facilitating the passage through the second replicative arrest. Accordingly, the chromosomal anomalies identified repeatedly in clones surviving the crisis might be important in cell immortalization. It is notable that in PE1-E6E7, the loss of chromosome 13 was present in the majority of cells surviving the crisis. Assessment of clonal evolution (Fig. 3) in PE1-E6E7 cells suggested the loss of chromosome 13 was an early event in those immortalized clones. Also, this was the only aberration in the line PE4-E6E7 and was present stably throughout the entire culture period. These observations suggest that the loss of chromosome 13 might have conferred a growth advantage during cell immortalization. The pathogenetic significance of loss of chromosome 13

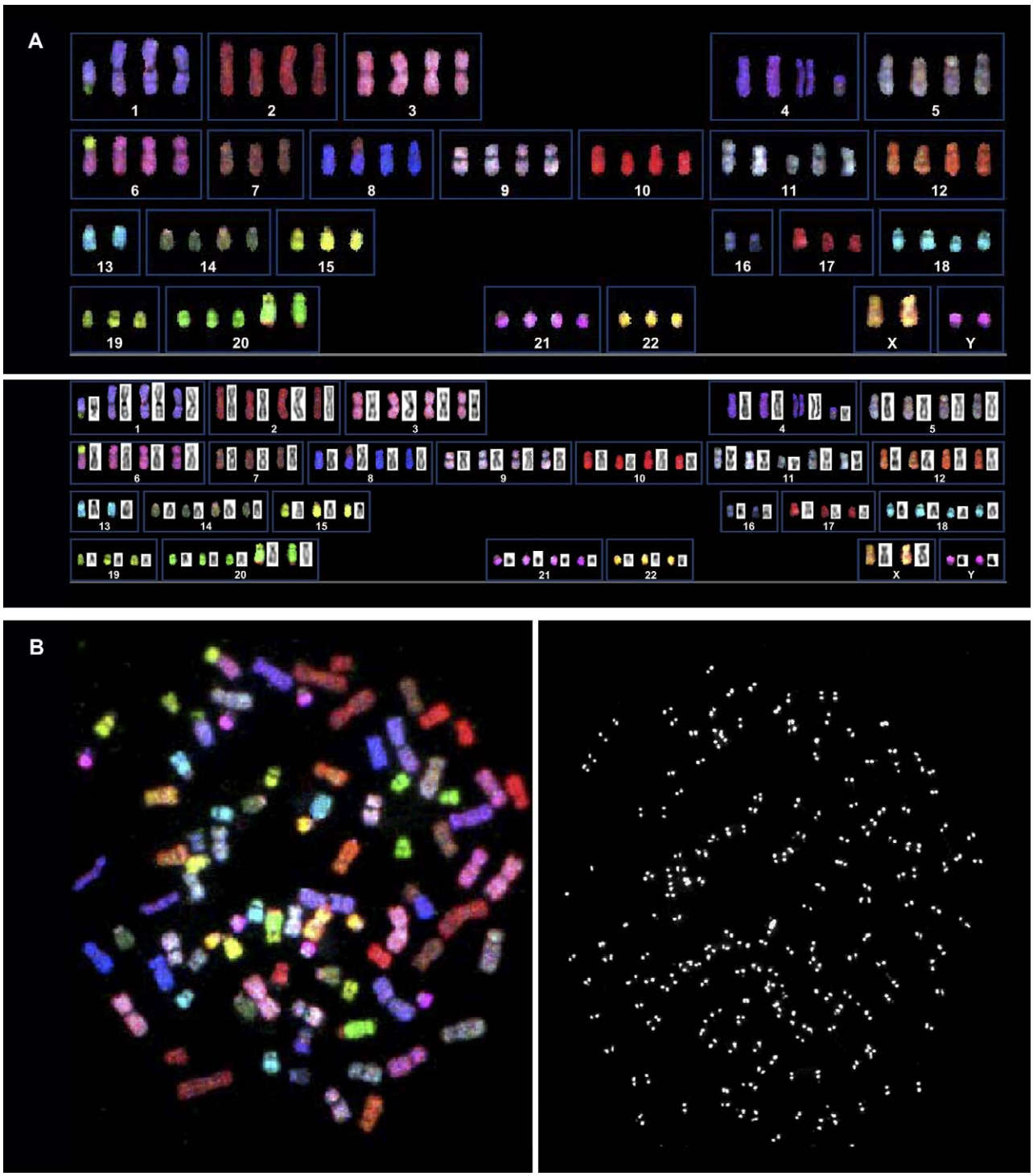

Fig. 3. PE1-E6E7 cells post-crisis at passage 71. (A) Spectral karyotyping confirmed a complex karyotype with a hypotetraploid modal chromosomal number. The complete karyotype was shown in Table 1. Note the loss of chromosomes 13 in this hypotetraploid karyotype. (B) A spectral karyotyped metaphase spread and the corresponding telomere fluorescence in situ hybridization showing strong and even signals at all chromosome ends.

in cell immortalization is further strengthened by recent studies showing that it was consistently observed in all five human ovarian surface epithelial cell lines (HOSE) after immortalization [10], as well as in seven of eight cultured human umbilical vein endothelial cells [11]. In the latter study, loss of chromosome 13 was observed as early as PD 20, and five strains became hypotetraploid shortly after the loss of chromosome 13. A review of the literature shows that loss of chromosome 13 is also a common finding in a broad spectrum of tumor types [12]. The nonrandom

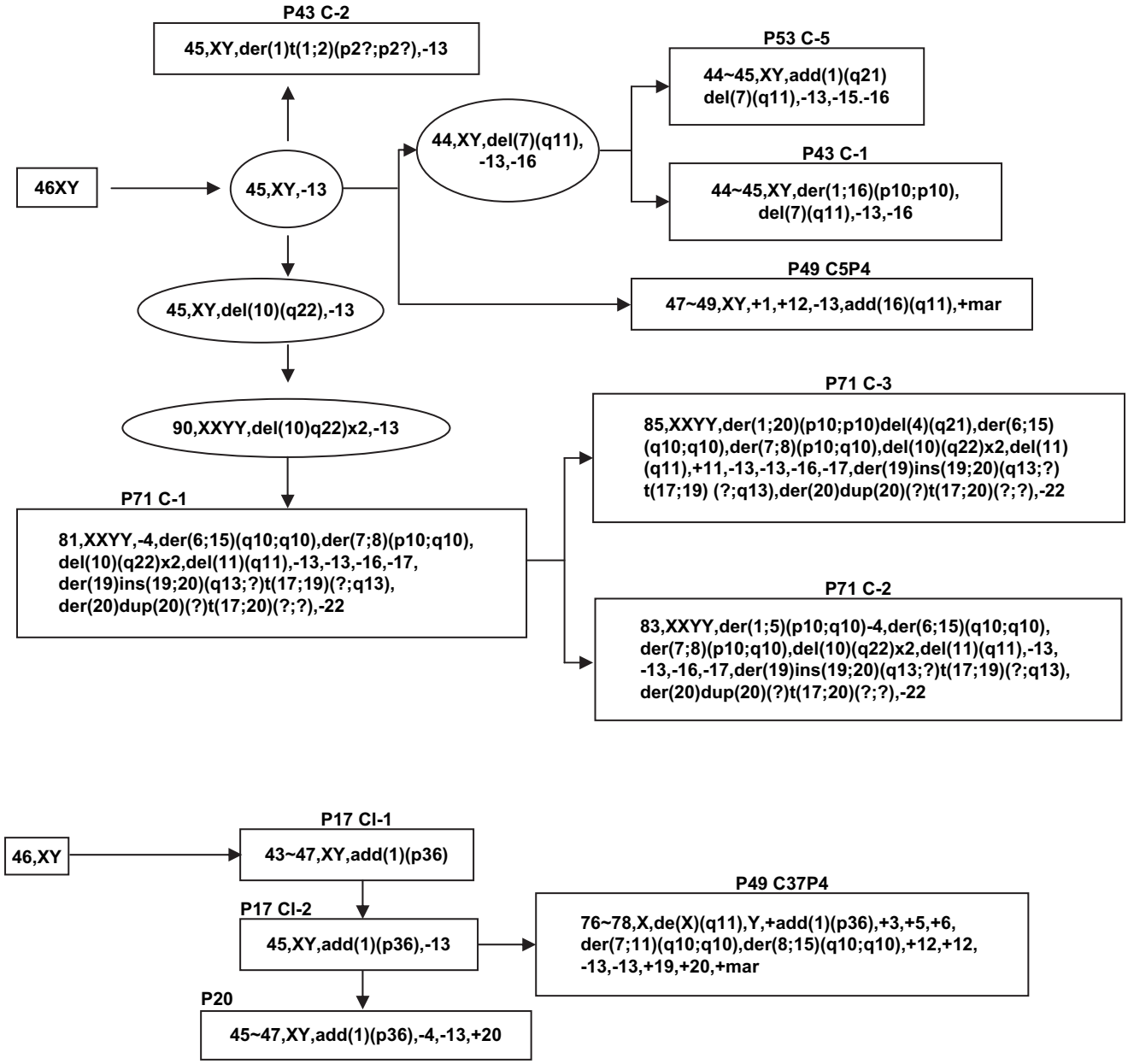

Fig. 4. Hypothetical karyotypic evolution of PE1-E6E7. Clones in boxes were observed, whereas clones in elipses were inferred, not observed.

occurrence of loss of chromosome 13 in immortalized cell lines and in various tumor types suggests that this may be a crucial step in early neoplastic transformation.

Genetic instability is an important feature of tumor cells, contributing to the step-wise accumulation of mutations leading to neoplastic transformation. Different lines of research have suggested recently that telomeric dysfunction might be crucial in chromosomal instability [13–16]. Indeed, mitotically unstable ring chromosomes, TA, dicentric chromosomes, and anaphase bridges, all putatively due to telomere shortening and the fusion of chromosomal ends, have been found in high frequency in benign and borderline malignant mesenchymal tumors, as well as in benign lesions in the head and neck region [17,18]. Furthermore, the observations of telomere dynamics, end-to-end fusions, and telomeric activation, as well as their relation to chromosome aberrations during the immortalization of other cell types transduced by SV-40 or E6E7, also supported this hypothesis [7,19,20]. In these studies, the mean telomere length was observed to decrease in pre-crisis cells, reaching a critical size during crisis corresponding to the highest frequency of TA and dicentric chromosomes that occur during this period. Our findings that the frequency of TA and dicentric chromosomes increased before crisis, peaked during crisis, and declined precipitously after crisis were in line with these studies. Furthermore, our findings also corroborated with our previous observations that telomeric dysfunction in the form of increased number of TTAGGG-negative

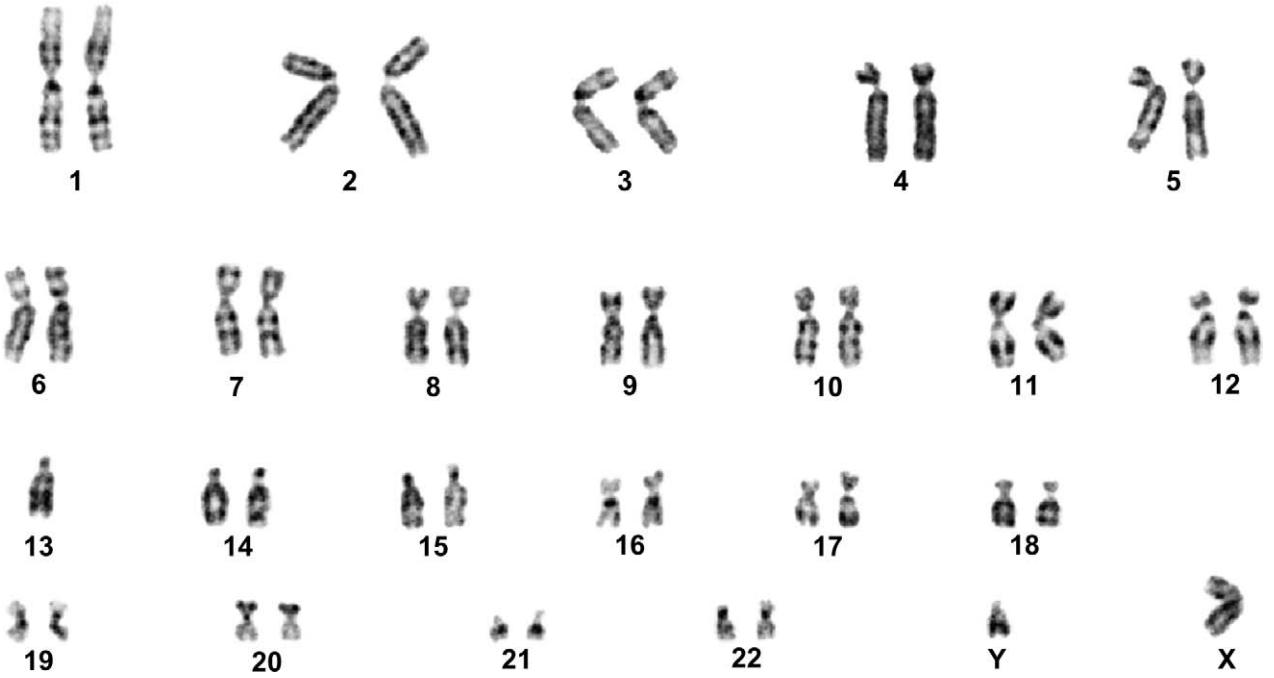

Fig. 5. A representative karyotype of PE4-E6E7. Loss of chromosome 13 is the sole karyotypic aberration.

termini, anaphase bridges, multipolar mitoses, and cells exhibiting supernumerary centrosomes were found in HOSE cells during and after crisis [19]. Therefore, telomeric dysfunction may be considered an important step leading to cellular immortalization and perhaps transformation.

Another interesting phenomenon is the frequent occurrence of centromeric rearrangements in the cells after immortalization. Centromeric and near-centromeric aberrations, often in the form of isochromsomes, whole-arm translocations, and deletions, are common finding in tumor cells. Little is known about the mechanisms or pathogenetic significance of these rearrangements. The findings in this study, as well as our observations in the immortalization of HOSE, nasopharyngeal, and esophageal cells [10,20], indicated that centromeric rearrangements were common. During cellular immortalization, there is a shift of aberration patterns from TA and dicentric chromosomes in crisis cells to the dominance of centromeric aberrations in post-crisis cells, suggesting a significant role of centromeric rearrangements in cell immortalization. More interestingly, our recent study of mitotic disturbance and dysfunction of telomeres in the crisis stage of HOSE cell lines has shown that the DNA breaks in chromosome bridges are preferentially located inside or adjacent to the alphoid sequences, resulting in the formation of stable whole-arm translocations and isochromosomes [19]. Together with findings in this study, these observations indirectly suggest that the breakage–fusion–bridge event triggered by the telomere dysfunction may be one of the mechanisms leading to centromeric aberrations. A schematic diagram of the sequence of genetic events leading to cellular immortalization is shown in Fig. 6. This may provide a hypothetical framework to be tested in future studies.

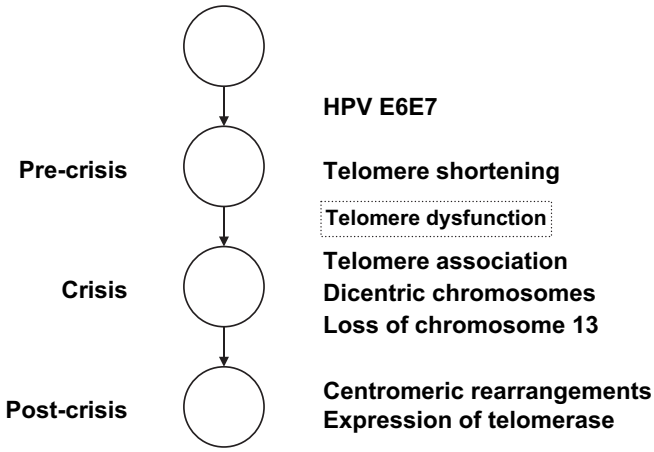

Fig. 6. Schematic diagram showing cytogenetic and molecular events in EVCT cell immortalization.

Acknowledgments

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