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Cytogenetic aberrations in immortalization of esophageal epithelial cells

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Abstract To define the early cytogenetic events important in esophageal carcinogenesis, we immortalized normal esophageal epithelial cells by overexpression of human papillomavirus type 16 E6/E7 (HPV16E6/E7) and human telomerase reverse transcriptase (hTERT), and characterized the chromosomal abnormalities serially before and after cellular immortalizaiton. During crisis, most cells had simple nonclonal karyotypic changes with cytogenetic divergence. Mitotically unstable chromosomes (i.e., telomere association and dicentric chromosomes) were the most common aberrations. After crisis, the karyotypic patterns were more convergent with nonrandom clonal changes. A few clones dominated the culture. Gain of chromosome 20q was consistently observed in four *HPVE6/E7* immortalized esophageal lines, whereas amplification of chromosome 5q was preferentially found in *hTERT* immortalized cells. In addition, chromosomal aberrations of immortalized cells, including del(3p) and centromere rearrangements, were similar to those observed in esophageal cancer. Furthermore, in E6/E7-expressing cells, the frequency of negative telomere termini and anaphase bridges were high during crisis and low after crisis. These findings suggested that telomere dysfunction might be an important cause of cellular crisis, and the resultant chromosomal aberrations, mainly amplification of chromosome 20q or 5q, might be early genetic events required in esophageal cell immortalization. These alterations might be valuable models for further study of molecular mechanisms contributing to esophageal carcinogenesis.

1. Introduction

Esophageal carcinoma is the ninth most common cancer worldwide and the second in China [1]. In Hong Kong, approximately 90% of esophageal carcinoma is squamous cell carcinoma (SCC), and the overall five-year survival rate remains low [2]. In Western countries, the incidence of esophageal adenocarcinoma has increased dramatically in recent years [3], with more than 85% of patients dying within 2 years of diagnosis [4].

Although esophageal cancer is an important malignancy, the genetic mechanisms underlying carcinogenesis have not been well defined, partly due to the difficulties in culturing esophageal tumor cells in vitro and the lack of suitable cell models. Cytogenetic information is available in 42 cases only [5]. Most of these tumors displayed complicated cytogenetic aberrations. Also, a few esophageal cancer cell lines available were mostly derived from advanced esophageal cancer and were characterized by highly complex karyotypes [6–9]. The relative importance of these complex aberrations in esophageal carcinogenesis remains undefined.

Recent studies have suggested that infection with the high-risk human papillomavirus type 16 E6/E7 (HPV16E6/E7) might play an important pathogenetic role in esophageal SCC in Chinese patients [10]. Progressive up-regulation of telomerase, however, appears to be the important feature of adenocarcinoma in the west [11]. Esophageal cells can be immortalized in vitro by the overexpression of the oncoprotein HPV16 E6/E7 or the catalytic subunit of human telomerase reverse transcriptase (hTERT) [12]. As immortalization may be an initial step of carcinogenesis, the study of cytogenetic changes associated with immortalization may help to define the genetic events critical for neoplastic transformation.

In this study, esophageal cell lines obtained by immortalization with transfection of the *HPV16E6/E7* and *hTERT* genes were characterized serially before and after

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immortalization. The karyotypic profiles of these cell lines were also compared with those of esophageal carcinoma cell lines. Finally, the relationship between the telomere status and chromosomal instability was evaluated in one of the *HPV16E6/E7*-expressing cell lines.

2. Materials and methods

2.1. Immortalization of normal esophageal epithelial cells

The method of primary culture and immortalization of normal epithelial cell lines [13] and the establishment and maintenance of the esophageal cancer cell lines HKESC-1 [6], KYSE-140 [8], SLMT-1 [9], EC-1, and EC-18 were as described previously. Five cell lines, four with the expression of *HPV16E6/E7*, NE108/E6E7, NE083/E6E7, NE3/E6E7, and NECA6/E6E7, and one with *hTERT* and NE083/hTERT, were newly immortalized.

2.2. Cytogenetic analysis

The cell cultures were harvested by conventional cytogenetic techniques. Briefly, cells were blocked at metaphase by exposure to colcemid (0.025 μ/mL; GIBCO, Invitrogen Corp., Paisley, UK) for 14 hours. After hypotonic and fixative treatment, the cell suspension was spread on slides, and the metaphase chromosomes were G-banded with Wright stain. The clonality and karyotypes were described according to the International System for Human Cytogenetic Nomenclature (1995) [14].

2.3. Fluorescence in situ hybridization (FISH)

Multicolor combined binary ratio labeling (COBRA) FISH was performed as described [15]. Briefly, slides with metaphase chromosomes were pretreated with pepsin and formaldehyde. Probe mix (8 μL) containing 24 human chromosomes labeled with four distinct fluorochromes by the combination of binary and ratio labeling was applied on the slide. The slide and probe were denatured simultaneously by incubation at 72°C on a hot plate. After hybridization for 48–72 hours at 37°C in a humid chamber, the slides were washed in 74°C 0.4× standard saline citrate (SSC) for 2 minutes. Chromosomes were counterstained by TNT (0.1 mol/L Tris, 0.15 mol/L NaCl, 0.05% Tween 20 in 100 mL of phosphate buffered saline) and 4',6-diamidino phenylindole (DAPI) (0.5 mg/mL) for 10 minutes. The slides were embedded in Citifluor (Ted Pella, Redding, CA) before microscopic evaluation. For analysis, an Axioplan-2 microscope (Carl Zeiss GmbH, Jena, Germany) coupled to a cooled charge-coupled device camera and a 12-position filter wheel was used. Acquired images were evaluated with the CytoVision ChromoFluor System (Applied Imaging, Newcastle, UK) [15].

Based on cytogenetic findings, specific bacterial artificial chromosomes (BAC) were selected for further FISH analysis (http://www.ncbi.nlm.nih.gov/cgi-bin/Etreez/mapsearch, 2005). The labeled probes were purified, precipitated, and dissolved in standard hybridization solution. The slide and probe were denatured simultaneously by incubation at 72°C on a hot plate. After hybridization overnight at 37°C in a humid chamber, the slides were washed in 74°C 0.4× SSC for 2 minutes, DAPI-stained, and analyzed.

Telomeric TTAGGG repeats were detected using telomeric FISH with a fluorescein-conjugated $(CCCTAA)_3$ peptide nucleic acid (PNA) probe. The number of negative chromosome termini for a metaphase cell was recorded. At least 20 cells were evaluated per case.

2.4. Mitotic cell morphology

For analysis of mitoses, cells cultured on chamber slides were washed in phosphate-buffered saline (PBS) for 5 minutes, fixed in methanol/acetic acid (3:1) at −20°C for 30 minutes, air-dried, and stained with hematoxylin (Accustain; Sigma, St. Louis, MO) and erythrosine (BDH, Poole, England). At least 50 anaphase cells were examined per population doublings (PD). An anaphase cell showing at least one string of chromatin connecting the poles was defined as harboring an anaphase bridge.

3. Results

3.1. Sequential cytogenetic characterization of immortalized normal esophageal cells

To identify cytogenetic changes potentially relevant to NE cell immortalization, sequential karyotyping was performed in four *HPV16E6/E7* and one *hTERT* immortalized cell line. The cytogenetic profiles of the five NE cell lines are summarized in Table 1.

3.2. NE108/E6E7

This line was thoroughly studied during immortalization. At PD21 (before crisis), most cells were karyotypically normal, although a few nonclonal chromosomal changes began to appear in 10.4% (5/48) of metaphase cells. At PD28–PD34 (crisis), cells with normal chromosome complement dropped to less than 50%, whereas cells harboring chromosome aberrations increased. At this stage, a considerable portion of metaphase cells showed nonclonal aberrations, but a few cytogenetically unrelated clones emerged. Chromosomal changes were highly heterogeneous, with nearly 30% of cells displaying mitotically unstable chromosomes [e.g., telomere association (tas), dicentric (dic), and ring (r) chromosomes]. Centromeric rearrangements (e.g., isochromosomes and whole-arm translocations) were also common. During immortalization (PD41–PD64), cells with related clonal chromosomal changes expanded, whereas