# Carcinogenesis and cellular immortalization without persistent inactivation of p16/Rb pathway in lung cancer

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**Abstract.** Existence of cancer stem cells (CSCs) is still hypothetical and their practical marker is not available yet in lung cancer. To verify the possible existence of CSCs and to find their markers in lung cancer, we compared the p16/Rb and telomerase status in 83 lung cancer tissues and 15 lung cancer cell lines, since inactivation of p16/Rb pathway is considered to be a prerequisite for normal somatic cells to become immortal cancer cells. We found that 7 of 14 adenocarcinoma, but not squamous cell carcinoma, tissues with high telomerase activity and 3 adenocarcinoma cell lines likely had intact p16/Rb. Such cell lines showed higher colony formation capacity in soft agar compared with inactivated ones with similar growth rate. Moreover, cisplatin-resistant cell line PC9/CDDP with intact p16/Rb, but not PC14/CDDP with its inactivation, increased the colony formation capacity compared with the parent cells. Since CSCs are considered to be resistant to conventional anticancer drugs, they could have been concentrated as long as CSCs existed. We propose that half of immortal lung adenocarcinomas are derived from innately telomerase-positive stem cells, which might be the origin of CSCs, and that high telomerase activity with intact p16/Rb could be a marker of stem cell origin.

## Introduction

The new concept describes a cancer stem cell (CSC) as a cell within a tumor that is able to self-renew and to produce the heterogenous lineages of cancer cells that comprise the tumor (1). Cancer stem cells have been speculated to be the source of many solid tumors including lung cancer (2), and be resistant to conventional chemo- and radio-therapy. Thus, identification of CSCs and their biomarkers in lung cancer is urgent to improve patient prognosis. Previously, CD133 (*PROM1*: prominin-1) positive cells (2,3), side population cells that extrude Hoechst 33342 dye (4), or aldehyde dehydrogenase positive cells (5) have been demonstrated to be the fractions of putative CSCs in malignant tumors including lung cancer, but they are still not conclusive (6).

It is widely accepted that CSCs have telomerase activity and are immortal so that they can produce cancer cells indefinitely (7-9). But it is not clear yet whether CSCs originate from normal stem cells or from differentiated somatic cells. Since human somatic cells are required to inactivate p16/Rb pathway to overcome cellular senescence and become immortal cancer cells concomitant with activation of telomerase (10,11), we hypothesized that immortal cancer cells without inactivation of p16/Rb pathway could not be derived from usual somatic cells, but be originated from innately telomerase-positive cells, i.e., stem cells. We previously found that all examined squamous cell carcinoma and small cell lung cancer (SCLC) tissues with high telomerase activity, meaning immortal cancer cells, had aberrations in *RB1* and/or *TP53* genes. However, in lung adenocarcinomas with high telomerase activity, neither gene was found in half of the samples (12). To verify this hypothesis, we examined p16 status in 83 lung cancer tissues and 15 lung cancer cell lines, and compared the relationship between the p16/Rb pathway status and the telomerase activity levels or colony formation capacities.

## Materials and methods

*Tumor samples*. A total of 83 surgically resected primary lung cancer tissues, including 42 adenocarcinomas, 30 squamous cell carcinomas, 4 adenosquamous cell carcinomas, and 7 SCLCs, were obtained from chemotherapy-naïve patients, as well as the corresponding adjacent non-cancer lung tissue samples as controls. All tissues had been provided by the Department of Pathology and Department of Molecular and Internal Medicine, Hiroshima University between 1991-1996. Their pathological stages had been assessed according to the International Staging System (13) and telomerase activity, *RB1* loss of heterozygosity (LOH), *TP53* LOH, and chromo-

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*Key words:* p16/Rb pathway, telomerase, cellular immortalization, cancer stem cells, lung cancer, adenocarcinoma

some 1p deletion mapping were previously reported (12,14-16). Written informed consent was obtained from all patients before surgery, and this study was approved by our Institutional Ethics Committee.

*Cell lines*. The SCLC cell line PC-6 and its SN-38- and CPT-11-resistant variants (SN2-5 and DQ2-2), a lung squamous cell carcinoma cell line LC-S, and lung adenocarcinoma cell lines PC-9 and PC-14 and their CDDP-resistant variants (PC-9/CDDP and PC-14/CDDP, respectively) as well as A549 were prepared and examined for CDDP sensitivity as previously described (17). The remaining 6 lung adenocarcinoma cell lines, RERF-LC-MS, VMRC-LCD, PC-3, RERF-LC-Ad1, RERF-LC-Ad2, and RERF-LC-KJ, and control fibroblast strain TIG-1 were obtained from the Health Science Research Resources Bank (Osaka, Japan).

*Colony formation assay with soft agar*. Anchorage-dependency of 11 lung adenocarcinoma cell lines was evaluated by conventional colony formation assay with soft agar in triplicate, as previous reported (18). Briefly, $5x10^3$ cells were cultured in 0.4% SeaPlaque GTG agarose (Bioproducts), and after 14 and 21 days of culture at 37˚C with 5% $CO_2$, colony number was counted under microscopy for slow-growing cells (colonies containing >5 cells were counted) and macroscopically with crystal violet staining for rapid growing cells (colonies macroscopically visible were counted).

*Preparation of DNA and RNA*. For tissue samples, genomic DNA had been prepared previously (15). For cell lines genomic DNA and total RNA were extracted from the frozen cell pellets using QIAamp™ DNA Mini kit (Qiagen Inc., Valencia, CA) and Qiagen RNeasy™ mini kit (Qiagen), respectively, according to the manufacturer's protocols.

*Real-time RT-PCR for evaluation of mRNA levels*. For cell lines, 2 $\mu$g of total RNA was reverse-transcribed using High-Capacity cDNA Archive™ Kit (Applied Biosystems, Foster City, CA, USA). An aliquot of the cDNA (equivalent to 10 ng total RNA) was subjected to each real-time RT-PCR using Universal Probe Library (UPL, Roche Diagnostics, Tokyo, Japan) for *CDKN2A* (p16), *PROM1*, *BMI1*, and *ABCG2*, TaqMan Gene Expression Assays for *RB1* (Hs00153108_m1 targeting exons 24-25, Applied Biosystems), and Pre-Developed TaqMan Assay Reagents (Applied Biosystems) for ACTB as an internal control. Each reaction was carried out in duplicate or triplicate using ABI PRISM™ 7900HT Sequence Detection System (Applied Biosystems) and relative gene expression levels calculated as ratio to *ACTB* expression level were standardized using a pooled cDNA derived from 17 various cancer cell lines. The UPL primers (final concentration 200 nM each) and MGB-probe (final concentration 100 nM) sets are as follows: *CDKN2A*-F: 5'-GTGGACCTGGCTGAG GAG-3'. *CDKN2A*-R: 5'-CTTTCAATCGGGGATGTCTG-3'. *CDKN2A*-probe: UPL no. 34 (Roche). *PROM1*-F: 5'-AACCT TACACGAGCAAGGAATTA-3'. *PROM1*-R: 5'-AAACTTG TTCAAAAGTGAGCTTCAT-3'. *PROM1*-probe: UPL no. 48 (Roche). *BMI1*-F: 5'-TTCTTTGACCAGAACAGATTGG-3'. *BMI1*-R: 5'-GCATCACAGTCATTGCTGCT-3'. *BMI1*-probe: UPL no. 63 (Roche). *ABCG2*-F: 5'- TGGCTTAGACTCAAG CACAGC-3'. *ABCG2*-R: 5'-TCGTCCCTGCTTAGACA TCC-3'. *ABCG2*-probe: UPL no. 56 (Roche).

*Real-time PCR for quantitation of DNA amounts*. Homozygous deletion of *CDKN2A* was determined by TaqMan™ quantitative real-time PCR system with TagMan Universal PCR master mix and the ABI PRISM™ 7900HT Sequence Detection System (Applied Biosystems) using *TFRC* as control gene amount (12). Using a 384-well reaction plate (Applied Biosystems), 20 ng of genomic DNA and 2 sets of primers (final concentration 200 nM each for *CDKN2A* and 300 and 900 nM for *TFRC* forward and reverse primers, respectively) and fluorescent-probes (final concentration 100 nM for FAM-labeled *CDKN2A* and 200 nM for VIC-labeled *TFRC*) were mixed in a 10-$\mu$l reaction mixture. The primer and probe set for *TFRC* was previously reported (12) and that for *CDKN2A* is as follows: *CDKN2A*-F: 5'-AGCTTCCTTTCCGTCA TGC-3'. *CDKN2A*-R: 5'-TCATGACCTGCCAGAGAGAA-3'. *CDKN2A*-probe: UPL no. 21 (Roche). Threshold cycle (Ct) for each gene was determined using thermocycler software and the average of 3 independent Cts was calculated. Standard DNA was prepared by mixing genomic DNAs derived from two samples, non-cancer cells with intact *CDKN2A* and a cancer cell line with homologous deletion, at 10:0 (STD-100%), 8:2 (-80%), 6:4 (-60%), 4:6 (-40%), 2:8 (-20%), and 0:10 (0%).

*Fragment analysis for detection of LOH*. LOH of *CDKN2A* was evaluated by fragment length analysis using microsatellite marker D9S1748. The FAM-labeled forward primer (5'-CACCTCAGAAGTCAGTGAGT-3') and a non-labeled reverse primer (5'-GTGCTTGAAATACACCTTTCC-3') were mixed with 20 ng of genomic DNA and subjected to PCR followed by fragment analysis using ABI PRISM 310 Genetic Analyzer and GeneScan™ software as previously described (12). The adjacent non-cancer lung tissue samples were used as controls of peak height ratio of the corresponding repeat numbers.

*Fragment analysis for quantitative methylation-specific PCR (qMSP)*. MSP was carried out to evaluate the *CDKN2A* (p16) CpG island methylation status according to a previous report (19) with quantitative modification. The bisulfite-treated genomic DNA was first amplified with outside non-fluorescent primers (35 cycles, annealing at 60˚C) and then nested PCR was carried out using FAM-labeled methylated or HEX-labeled unmethylated allele-specific primers with annealing temperature at 65˚C. Then, 0.5 $\mu$l each of methylated (FAM labeled) and unmethylated (HEX labeled) PCR products were mixed and subjected to ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The methylation ratio was calculated as (area of FAM peak)/(areas of FAM peak + HEX peak).

*Direct sequence analysis for RB1 cDNA*. cDNAs derived from the 15 lung cancer cell lines were subjected to PCR using 2 sets of primers amplifying exons 10-26 that cover almost all naturally occurring *RB1* mutations. The PCR condition was 95˚C for 15 min followed by 40 cycles of 95˚C for 30 sec, 58˚C for 1 min, and 72˚C for 90 sec. Sequence analyses were carried out using Big Dye Terminator v1.1 Cycle Sequencing

Table I. The genetic aberrations in 83 primary lung cancers.

| No. | Gender | Age | Stage | Tel[a] | *TP53*[a] | *RB1*[a] | 1p34[a] | *CDKN2A* HD/LOH | *CDKN2A* qMSP | p16/Rb inactivation |
|---|---|---|---|---|---|---|---|---|---|---|
| Adenocarcinoma | | | | | | | | | | |
| 1 | F | 61 | I | Low | Hetero | Hetero | Hetero | Hetero | 1.0 | + |
| 2 | M | 69 | I | Low | Hetero | Hetero | Hetero | Hetero | 1.0 | + |
| 3 | M | 75 | I | Low | Hetero | Hetero | Hetero | Hetero | 1.0 | + |
| 4 | M | 62 | I | Low | Hetero | Hetero | Hetero | Hetero | 1.0 | + |
| 5 | M | 66 | I | Low | Hetero | Hetero | Hetero | Hetero | 1.0 | + |
| 6 | F | 67 | I | Low | Hetero | Hetero | Hetero | Hetero | 0.80 | + |
| 7 | F | 67 | II | Low | Hetero | Hetero | Hetero | Hetero | 1.0 | + |
| 8 | M | 76 | IIIA | Low | Hetero | Hetero | Hetero | Hetero | 0.99 | + |
| 9 | M | 61 | IIIA | Low | Hetero | Hetero | Hetero | NI | 1.0 | + |
| 10 | M | 60 | IIIB | Low | Hetero | Hetero | Hetero | Hetero | 0.27 | + |
| 11 | F | 64 | IV | Low | Hetero | Hetero | Hetero | HD | 0 | + |
| 12 | F | 56 | IV | Low | Hetero | Hetero | Hetero | NI | 0.59 | + |
| 13 | F | 57 | IIIA | Low | Hetero | Hetero | LOH | NI | 1.0 | + |
| 14 | M | 60 | IV | Low | Hetero | Hetero | LOH | Hetero | 1.0 | + |
| 15 | F | 65 | IV | Low | Hetero | Hetero | LOH | LOH | 0.88 | + |
| 16 | F | 62 | I | Low | Hetero | LOH | LOH | Hetero | 0.28 | + |
| 17 | F | 60 | I | Low | NI | Hetero | Hetero | Hetero | 0.90 | + |
| 18 | M | 48 | I | Low | NI | Hetero | LOH | NI | 1.0 | + |
| 19 | F | 51 | IIIa | Low | NI | Hetero | Hetero | Hetero | 1.0 | + |
| 20 | F | 57 | I | Low | LOH | Hetero | LOH | LOH | 0.69 | + |
| 21 | M | 58 | IV | Low | LOH | Hetero | Hetero | Hetero | 1.0 | + |
| 22 | F | 56 | IV | Low | LOH | Hetero | Hetero | Hetero | 1.0 | + |
| 23 | M | 66 | IV | Low | LOH | Hetero | Hetero | Hetero | 1.0 | + |
| 24 | F | 72 | IIIA | Low | LOH | LOH | Hetero | Hetero | 0.97 | + |
| 25 | M | 67 | I | Low | LOH | LOH | LOH | Hetero | 0.94 | + |
| 26 | M | 77 | I | Low | LOH | LOH | LOH | NI | 0.11 | + |
| 27 | M | 64 | I | Low | LOH | LOH | LOH | Hetero | 0.84 | + |
| 28 | M | 62 | IIIA | Low | LOH | LOH | LOH | NI | 0.10 | + |
| 29 | F | 78 | I | High | Hetero | Hetero | Hetero | Hetero | 0 | - |
| 30 | M | 69 | I | High | Hetero | Hetero | Hetero | Hetero | 0.87 | + |
| 31 | M | 62 | I | High | Hetero | Hetero | Hetero | Hetero | 0.11 | - |
| 32 | M | 75 | I | High | Hetero | Hetero | Hetero | NI | 0 | - |
| 33 | F | 62 | II | High | Hetero | Hetero | Hetero | Hetero | 0 | - |
| 34 | F | 69 | IIIA | High | Hetero | Hetero | Hetero | Hetero | 0.07 | - |
| 35 | F | 77 | IV | High | Hetero | Hetero | Hetero | HD | 0 | + |
| 36 | F | 80 | I | High | Hetero | LOH | Hetero | Hetero | 0 | + |
| 37 | F | 59 | IIIA | High | LOH | Hetero | Hetero | Hetero | 0.13 | - |
| 38 | M | 76 | IIIA | High | LOH | Hetero | LOH | Hetero | 0.15 | - |
| 39 | M | 55 | I | High | LOH | LOH | LOH | HD | 0 | + |
| 40 | M | 68 | II | High | LOH | LOH | LOH | Hetero | 0.10 | + |
| 41 | M | 66 | IV | High | LOH | LOH | Hetero | Hetero | 0.12 | + |
| 42 | M | 50 | IIIB | High | LOH | LOH | LOH | Hetero | 0 | + |
| Adenosquamous cell carcinoma | | | | | | | | | | |
| 43 | M | 75 | IIIA | Low | Hetero | Hetero | Hetero | HD | 0 | + |
| 44 | M | 42 | I | Low | NI | LOH | Hetero | NI | 1.0 | + |
| 45 | M | 75 | IV | Low | LOH | LOH | Amp | LOH | 1.0 | + |
| 46 | M | 69 | IIIA | High | LOH | LOH | LOH | HD | 0.21 | + |

Table I. Continued.

| No. | Gender | Age | Stage | Tel[a] | *TP53*[a] | *RB1*[a] | 1p34[a] | *CDKN2A* | | p16/Rb inactivation |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | HD/LOH | qMSP | |
| Small cell lung cancer | | | | | | | | | | |
| 47 | M | 75 | I | Low | LOH | Hetero | LOH | Hetero | 0 | - |
| 48 | F | 79 | II | High | LOH | Hetero | Hetero | Hetero | 0 | - |
| 49 | M | 56 | I | High | LOH | LOH | Amp | Hetero | 0 | + |
| 50 | M | 71 | IIIA | High | LOH | LOH | Amp | Hetero | 0.28 | + |
| 51 | M | 77 | IIIA | High | LOH | LOH | Hetero | Hetero | 0 | + |
| 52 | M | 74 | I | High | LOH | LOH | LOH | HD | 0 | + |
| 53 | M | 74 | II | High | LOH | LOH | LOH | Hetero | 0 | + |
| Squamous cell carcinoma | | | | | | | | | | |
| 54 | M | 74 | I | Low | Hetero | Hetero | Hetero | NI | 0 | - |
| 55 | F | 62 | I | Low | Hetero | Hetero | LOH | Hetero | 0 | - |
| 56 | M | 70 | II | Low | Hetero | Hetero | Hetero | NI | 0 | - |
| 57 | M | 69 | II | Low | Hetero | Hetero | Hetero | NI | 0 | - |
| 58 | M | 63 | II | Low | Hetero | Hetero | Hetero | Hetero | 0 | - |
| 59 | M | 52 | IIIA | Low | Hetero | Hetero | Hetero | Hetero | 0 | - |
| 60 | M | 79 | IIIA | Low | Hetero | Hetero | Hetero | Hetero | 0 | - |
| 61 | M | 70 | IIIA | Low | Hetero | Hetero | Hetero | LOH | 0 | + |
| 62 | M | 69 | IIIA | Low | Hetero | LOH | Hetero | LOH | 0 | + |
| 63 | M | 77 | IIIB | Low | Hetero | LOH | LOH | Hetero | 0 | + |
| 64 | M | 61 | II | Low | NI | Hetero | LOH | Hetero | 0 | - |
| 65 | M | 72 | I | Low | LOH | Hetero | Hetero | Hetero | NI | - |
| 66 | M | 67 | I | Low | LOH | Hetero | Hetero | LOH | 0 | + |
| 67 | M | 60 | IV | Low | LOH | Hetero | LOH | NI | 0 | - |
| 68 | M | 71 | I | Low | LOH | Hetero | LOH | HD | 0 | + |
| 69 | M | 73 | I | Low | LOH | LOH | Hetero | HD | 0 | + |
| 70 | M | 87 | I | Low | LOH | LOH | Hetero | Hetero | 0 | + |
| 71 | M | 65 | IIIA | Low | LOH | LOH | Hetero | LOH | 0 | + |
| 72 | F | 71 | I | Low | LOH | LOH | LOH | Hetero | 0 | + |
| 73 | M | 63 | I | Low | LOH | LOH | LOH | LOH | 0 | + |
| 74 | M | 64 | IIIA | Low | LOH | LOH | LOH | HD | NI | + |
| 75 | M | 73 | IV | Low | LOH | NI | Hetero | Hetero | 0 | - |
| 76 | M | 66 | I | Low | LOH | NI | LOH | Hetero | 0 | - |
| 77 | M | 65 | IIIA | Low | LOH | NI | LOH | LOH | 0.02 | - |
| 78 | M | 72 | IIIB | High | Hetero | LOH | LOH | LOH | 0 | + |
| 79 | M | 62 | IIIB | High | Hetero | LOH | LOH | Hetero | 0 | + |
| 80 | M | 67 | IIIA | High | LOH | Hetero | LOH | HD | 0 | + |
| 81 | M | 62 | IIIA | High | LOH | LOH | Hetero | HD | 0 | + |
| 82 | M | 53 | IV | High | LOH | LOH | LOH | NI | 0 | + |
| 83 | M | 71 | I | High | LOH | NI | LOH | HD | NI | + |

[a]Previously analyzed for Tel (telomerase activity level), *TP53* (LOH in *TP53*), *RB1* (LOH in *RB1*), 1p34 (deletion within 1p34 to pter) (12,14-16). qMSP, (methylated allele peak)/(methylated + unmethylated allele peaks) area ratio >0.25 was considered as methylated; HD, homologous deletion; NI, not informative.

Kit™ and ABI PRISM 310 or PRISM 3100 Genetic Analyzer (Applied Biosystems). The 2 set primers are: *RB1*set1ex9-F: 5'-CTAATGGACTTCCAGAGGTTG-3'. *RB1*set1ex20-R: 5'-CGGAGATAGGCTAGCCGATA-3'. *RB1*set2ex19-F:

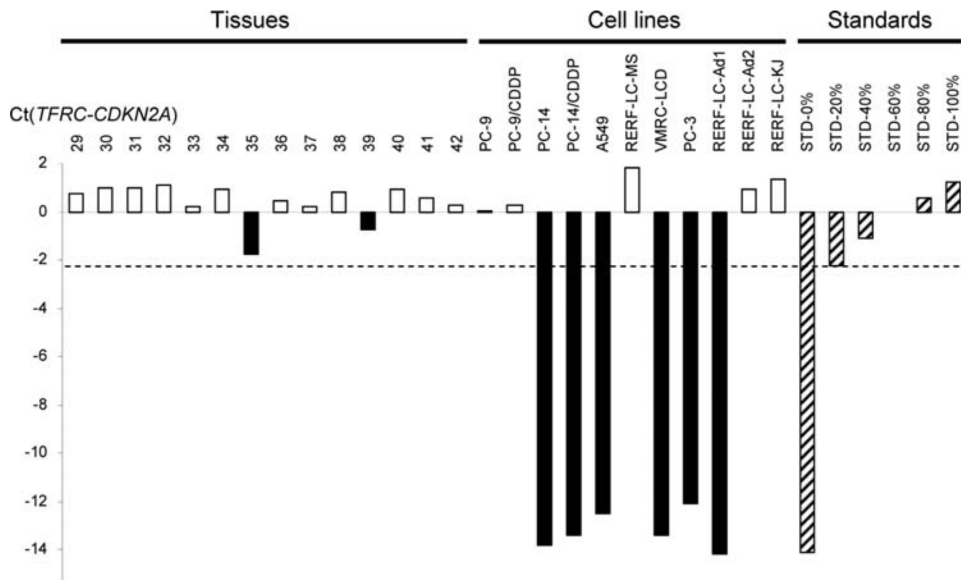


Figure 1. Detection of homologous deletion of p16 by quantitative real-time PCR for *CDKN2A* using control gene *TFRC* in representative samples (all adenocarcinoma tissues with high telomerase activity and lung adenocarcinoma cell lines). Tissue samples were classified as having homologous deletion when Ct(*TFRC*)-Ct(*CDKN2A*) in each sample < STD-60% (= 0, smooth line), and cell lines when < STD-20% (= -2.25, dotted line). Ct, threshold cycles; Hatched bar, standard DNA for quantitation; Closed bar, classified as homologous deletion; Open bar, classified as retaining at least one allele.

5'-TACTGCAAATGCAGAGACAC-3'. *RB1*set2ex27-R: 5'-GAAGAGGAAACAATCTGCTA-3'.

*Telomerase activity and other genetic aberrations*. Among the genetic aberrations in the tissue samples summarized in Table I, we previously evaluated and reported the telomerase activity level by TRAP assay (14), 1p34 LOH by deletion mapping using 12 polymorphic markers (16), and LOH of *TP53* and *RB1* genes using 5 and 4 polymorphic markers, respectively (12,15).

*Statistical analysis*. All statistical tests were performed using StatView version 5.0 software (SAS Institute Inc., Cary, NC, USA), and the Student's t-test, $\chi^2$, or Fisher's exact test was used to determined the P-value. Differences of P<0.05 were considered statistically significant.

## Results

Telomerase activity levels and LOH of *TP53*, *RB1*, and 1p34 locus in the lung cancer tissues have been evaluated previously (12,14-16) and summarized in Table I with the present data.

*p16 inactivation*. Persistent type inactivation of p16 was evaluated by the existence of *CDKN2A* homozygous deletion, LOH, and/or promoter methylation in 83 lung cancer tissues, and by *CDKN2A* homozygous deletion or complete promoter methylation in 15 lung cancer cell lines.

Homozygous deletion, [threshold cycles (Ct) of *TFRC*]-(Ct of *CDKN2A*) was smaller in a sample than that of 60% (for tissue samples) or 20% (for cell lines) standards in real-time PCR considering the existence of contaminated non-cancer cells in tissue samples, was found in 3 adenocarcinoma, 6 squamous cell carcinoma, 2 adenosquamous cell carcinoma, and 1 SCLC tissues and 6 adenocarcinoma cell lines (Fig. 1) and 1 squamous cell carcinoma cell line, LC-S. LOH, i.e., the calculated peak height ratio of the 2 alleles was <70% or >150% of that in normal counterpart in fragment analysis, was found in 10 tissue samples (Fig. 2). Cell lines could not be analyzed for LOH due to the lack of normal counterpart.

For evaluating *CDKN2A* promoter methylation, we considered it methylated when the methylated allele ratio, (methylated allele peak area)/(methylated and unmethylated allele peak areas), was >25%, and complete methylation when the ratio was >80% (Fig. 3). The *CDKN2A* promoter methylation was detected in 61.9% (26/42) of adenocarcinoma, but none of squamous cell carcinomas. For lung cancer cell lines, RERF-LC-MS was completely methylated among the 8 cell lines that retained *CDKN2A* gene. Thus, p16 inactivation by deletion or promoter methylation is summarized in Tables II and III, and such inactivation in adenocarcinoma tissues revealed to be highly associated with low/nil telomerase activity (P=0.0001).

*Rb inactivation*. Inactivation of Rb was evaluated by the existence of *RB1* LOH (analyzed for 1 RFLP and 3 microsatellite loci) in 83 lung cancer tissues and by the existence of genetic aberrations in *RB1* cDNA (sequence analysis of exons 10-27, that cover the mutation hot-spot region) or absence of *RB1* mRNA detection (real-time RT-PCR) in the 15 lung cancer cell lines.

For the 83 lung cancer tissue samples, we previously analyzed and reported that *RB1* LOH was found in 26 (34.2%) of 76 non-small cell lung cancer (NSCLC) and 5 (71.4%) of 7 SCLC tissues (12,15). Among the 15 lung cancer cell lines examined, *RB1* expression could not be detected in 1 adenocarcinoma cell line, RERF-LC-KJ (Fig. 4A). Also by sequence analysis of *RB1* cDNA, only this cell line was revealed to have an aberration, an inframe deletion of 174 nucleotides by splicing out the entire exons 24 and 25.

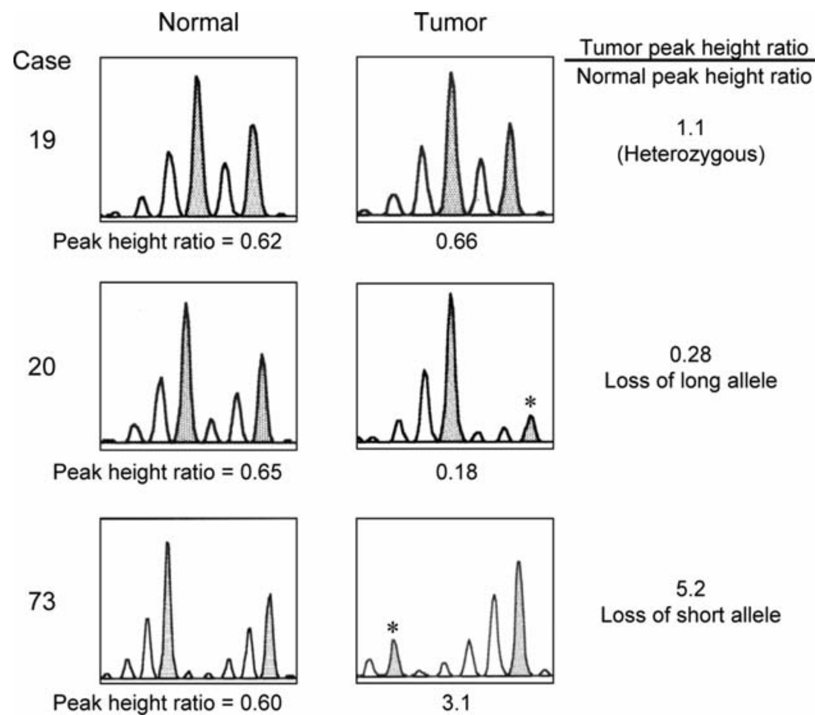


Figure 2. Detection of LOH of p16 by fragment analysis in representative samples. LOH, in cancer tissues was defined when the peak height ratio of heterozygous bands was <0.7 or >1.5 of expected ratio calculated from the normal counterpart. *Deleted allele demonstrating low peak possibly derived from contaminated non-cancer cells.

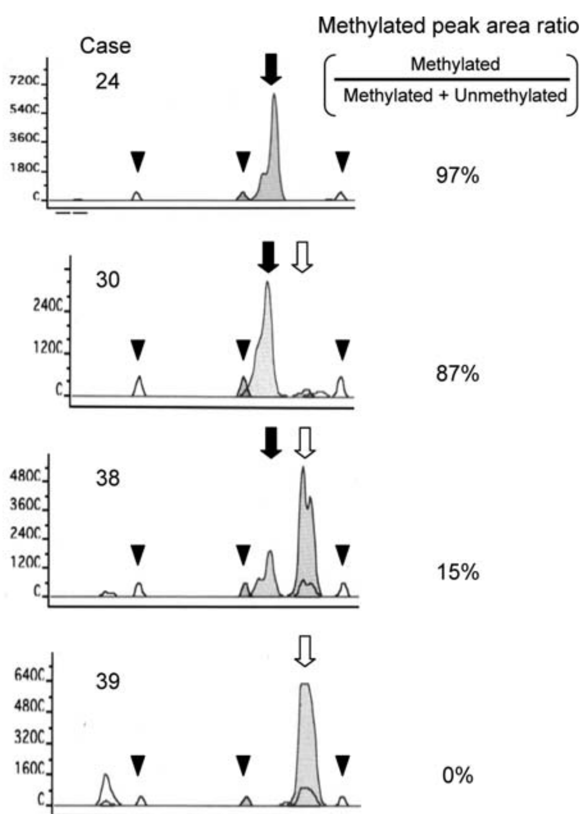


Figure 3. Quantitative evaluation of promoter methylation of p16 by fragment analysis in representative adenocarcinoma tissues. Methylated allele was labeled with FAM (closed arrow) and unmethylated allele was labeled with HEX (open arrow). The methylation ratio was calculated as (area of FAM peak)/(sum of areas of FAM & HEX peaks), and >25% was considered to be methylated and >80% as completely methylated. Arrowhead, size marker.

*Inactivation of p16/Rb pathway*. Combining above, inactivation of p16/Rb pathway, i.e., deletion/methylation of p16 and/or LOH of *RB1*, among the 83 lung cancer tissues was found in all 28 (100%) with low/nil telomerase activity but in only 7 of 14 (50%) with high telomerase activity (P=0.0001) in adenocarcinomas, whereas this relationship was the opposite in the remaining histology tumors (P=0.0309) (Table II). The p16 inactivation was inversely correlated with *RB1* LOH in adenocarcinoma tissues (P=0.0488).

Among the 15 lung cancer cell lines, the Rb inactivated cell line RERF-LC-KJ showed intact p16 as expected. Among the 11 adenocarcinoma cell lines, the p16/Rb pathway was considered to be intact in PC-9, its CDDP resistant variant PC-9/CDDP, and RERF-LC-Ad2 (p16/Rb pathway intact group) and inactivated in the remaining 8 cell lines (inactivated group). Then, cellular growth rate and colony formation capacity were carried out between these 2 groups.

*Expression of cancer stem cell markers*. As known cancer stem cell markers, we evaluated mRNA expression levels of *PROM1* (CD133), *BMI1*, and *ABCG2* by real-time RT-PCR in the 15 lung cancer cell lines. While the expression levels of *BMI1* and *ABCG2* genes were comparable among the all cell lines except for *ABCG2* in the drug resistant SCLC clones, *PROM1* was highly expressed in RERF-LC-Ad2, in which the p16/Rb pathway was considered to be intact (Fig. 4B).

*Growth rate of adenocarcinoma cell lines*. We found that the 11 adenocarcinoma cell lines can be divided into 2 groups according to their growth rates: >20 times multiplied at day 5 (rapid growth group, Fig. 5A) and less than that (slow growth group, Fig. 5B). The p16/Rb-intact cell lines PC-9 and PC-9/

Table II. Inactivation of p16/Rb in 83 lung cancer tissues.

| Histology | Telomerase activity[a] | *RB1* LOH[a] | p16 inactivation | p16 and/or Rb inactivation |
|---|---|---|---|---|
| Adenocarcinoma | | | | |
| | High (n=14) | 5 (35.7%) | 3 (21.4%) | 7 (50%) |
| | Low/nil (n=28) | 6 (21.4%) | 26 (92.9%) | 28 (100%) |
| Adenosquamous cell carcinoma | | | | |
| | High (n=1) | 1 (100%) | 1 (100%) | 1 (100%) |
| | Low/nil (n=3) | 2 (66.7%) | 3 (100%) | 3 (100%) |
| Squamous cell carcinoma | | | | |
| | High (n=6) | 4 (66.7%) | 4 (66.7%) | 6 (100%) |
| | Low/nil (n=24) | 8 (33.3%) | 9 (37.5%) | 12 (50.0%) |
| Small cell lung cancer | | | | |
| | High (n=6) | 5 (83.3%) | 2 (33.3%) | 5 (83.3%) |
| | Low/nil (n=1) | 0 (0%) | 0 (0%) | 0 (0%) |

[a]Previously analyzed for telomerase activity level and *RB1* LOH (12,14-16).

Table III. Inactivation of p16/Rb in lung cancer cell lines.

| | Rb inactivation | | p16 inactivation | | |
|---|---|---|---|---|---|
| Histology | RT-PCR[a] | Deletion | HD | qMSP | p16/Rb inactivation |
| Adenocarcinoma | | | | | |
| PC-9 | - | - | - | 0.46 | - |
| PC-9/CDDP | - | - | - | 0.40 | - |
| PC-14 | - | - | + | NI | + |
| PC-14/CDDP | - | - | + | NI | + |
| A549 | - | - | + | NI | + |
| RERF-LC-MS | - | - | - | 1.0 | + |
| VMRC-LCD | - | - | + | NI | + |
| PC-3 | - | - | + | NI | + |
| RERF-LC-Ad1 | - | - | + | NI | + |
| RERF-LC-Ad2 | - | - | - | 0 | - |
| RERF-LC-KJ | + | + | - | 0 | + |
| Squamous cell carcinoma | | | | | |
| LC-S | - | - | + | NI | + |
| Small cell lung cancer | | | | | |
| PC-6 | - | - | - | 0.01 | - |
| SN2-5 | - | - | - | 0.04 | - |
| DQ2-2 | - | - | - | 0.16 | - |

[a]RT-PCR +, not detectable by real-time RT-PCR; qMSP, methylation ratio >0.8 was considered as completely inactivated; HD, homologous deletion; NI, not informative.

CDDP in the former group and RERF-LC-Ad2 in the latter group did not show accelerated growth compared to the inactivated ones in each group.

*Colony formation assay with soft agar*. While the colonies in the rapid growth group were evaluated macroscopically after crystal violet staining (Figs. 5C and 6), those in the slow

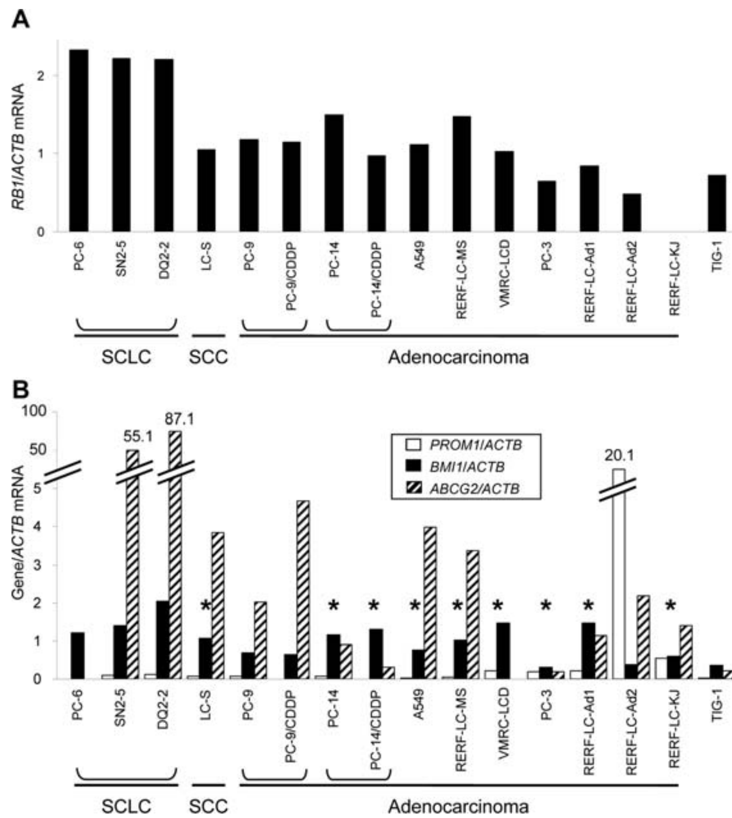


Figure 4. mRNA expression levels in all 15 cell lines evaluated by real-time RT-PCR using *ACTB* as internal control. *RB1* was not detected in RERF-LC-KJ (A). *PROM1* (open bar) was highly expressed in RERF-LC-Ad2, while the expression levels of *BMI1* (closed bar) and *ABCG2* (hatched bar) genes were comparable except for the drug resistant SCLC clones for *ABCG2* (B). Cell lines inactivated for p16/Rb pathway (homologous deletion or complete methylation of p16 or complete inactivation of *RB1*) are indicated by asterisk. TIG-1 is a non-cancer control human fibroblasts.

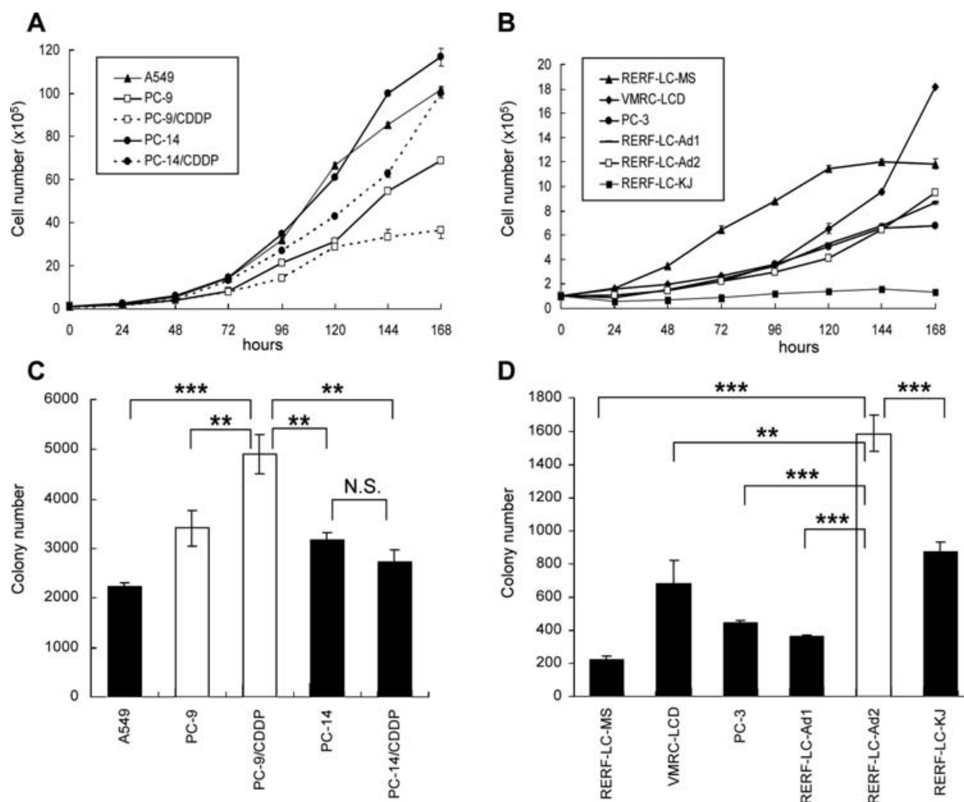


Figure 5. Growth rate and colony numbers in colony formation assay for the 11 lung adenocarcinoma cell lines. In each 60-mm diameter dish, $10^5$ each of rapid growth cell lines (A) or slow growth cell lines (B) were cultured, and cell number was calculated every day for 1 week in triplicate. Colony numbers of the rapid growth cell lines were evaluated macroscopically after crystal violet staining (C) while those of the slow growth cell lines were evaluated microscopically (D). p16/Rb intact cell lines (open box) did not show higher growth but showed increased colonies (open bar) in soft agar compared to p16/Rb inactivated cell lines (closed bar) in each group. **P<0.01; ***P<0.001; N.S., not significant.

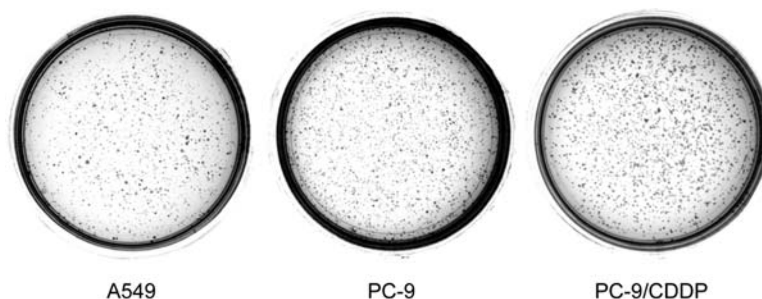


Figure 6. Colony formation assay for representative adenocarcinoma cell lines with rapid growth. Macroscopically visible colonies shown after crystal violet staining are p16/Rb inactivated cell line A549 < p16/Rb intact PC-9 < CDDP resistant PC-9 variant PC-9/CDDP.

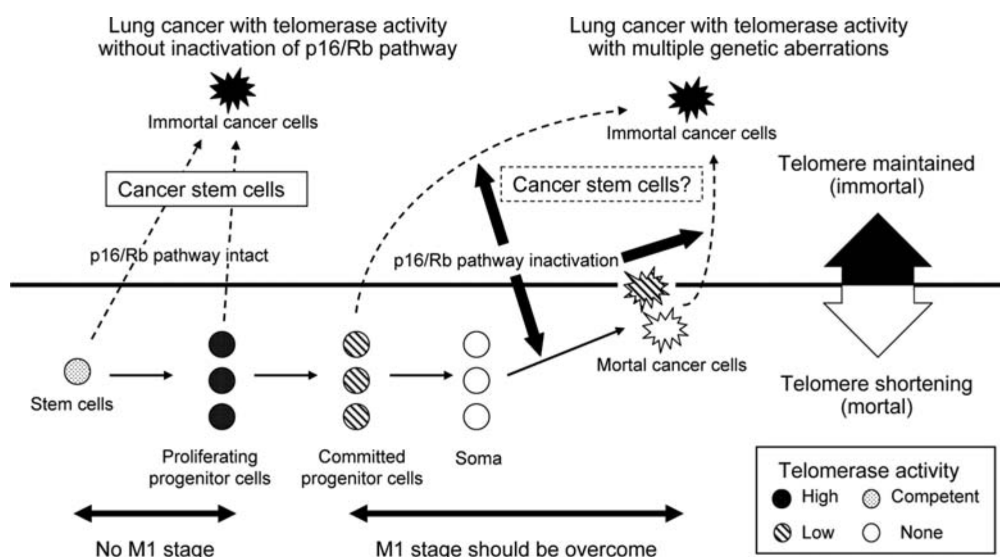


Figure 7. Our hypothesis of lung carcinogenesis. Immortal lung cancer cells might be derived from either of the two pathways: developed from telomerase positive normal cells, i.e., stem cells, through cancer stem cells or from telomerase negative somatic cells through multiple clonal selections acquiring genetic aberrations including *TP53*, *CDKN2A*, and *RB1* inactivations and telomerase activation, but not necessarily through cancer stem cells. Half of lung adenocarcinomas with high telomerase activity may develop from the former pathway, while most of squamous cell carcinomas develop from the latter pathway. M1, mortality stage 1; M2, mortality stage 2.

growth group were evaluated microscopically before staining, since they were small and macroscopically invisible in general even after 3 weeks (Fig. 5D). The p16/Rb intact cell lines, PC-9 and PC-9/CDDP in the rapid growth group and RERF-LC-Ad2 in the slow growth group, showed significantly higher colony formation capacity than the p16/Rb inactivated cell lines in each group (P<0.01-P<0.0001). Moreover, CDDP-resistant clone with intact p16/Rb (PC-9/CDDP), but not that with p16/Rb inactivation (PC-14/CDDP), showed significantly higher colony formation capacity than the parent clone (P<0.001, Figs. 5C and 6).

## Discussion

Most malignant tumors must have a mechanism for bypassing senescence to acquire the unlimited proliferative capacity that is required for advanced cancers. Human somatic cells are considered to have two stages of checkpoint before acquiring immortality (two-stage model for cellular senescence): mortality stage 1 (M1) that can be overcome by inactivation of p16/Rb and p53 pathways and mortality stage 2 (M2), that requires activation of telomerase to be overcome (11,20). In fact, we previously found that all examined lung squamous cell carcinoma and SCLC tissues with high telomerase activity had aberrations in *RB1* and/or *TP53* genes. However, in lung adenocarcinomas with high telomerase activity, neither gene was found in half of them (12). We also showed that cancer tissues with high telomerase activity consisted of predominantly telomerase positive cells, i.e., immortal cancer cells, while those with low telomerase activity consisted of predominantly telomerase negative cells (21), and lung cancer cell lines always showed high telomerase activity (14). In addition, we confirmed full-length type *TERT* mRNA expression in all 15 lung cancer cell lines used in the present study by real-time RT-PCR, which had been confirmed to correlate with telomerase activity (22). Since we previously found that lung cancers which might have originated from telomerase positive bronchial epithelia always showed high telomerase activity (23), lung cancers with high telomerase activity, consist of predominantly immortal cancer cells, and immortal lung cancer cell lines might have been derived from either of the two pathways:

developed from telomerase positive normal cells, i.e., stem cells, or from telomerase negative somatic cells through multiple clonal selections acquiring genetic aberrations including *TP53*, *CDKN2A*, and/or *RB1* inactivation and telomerase activation.

To confirm this hypothesis, we examined incidence of persistent type p16 inactivation (deletion and/or methylation of *CDKN2A*) in the tissue samples as well as p16/Rb status and colony formation capacity in cell lines in the present study. It has been reported that the inactivation of p16 occurs most prominently through promoter methylation or homozygous deletion and less often through mutation in lung cancers (24). As we speculated, persistent inactivation of p16/Rb pathway, deletion of *CDKN2A* or *RB1* or methylation of *CDKN2A* promoter, was found in all adenocarcinoma tissues with low/nil telomerase activity, while neither was found in half of adenocarcinomas with high telomerase activity (P=0.0001). For other genetic aberrations, we previously reported that the *RB1* LOH was associated with *TP53* and 1p34 LOH but not with *EGFR* aberrations (12). In the present study, 5 of 7 p16/Rb intact adenocarcinomas with high telomerase activity also lacked the *TP53* LOH and 4 of them lacked both *TP53* and 1p34 LOH (Table I), indicating that these cases have escaped the M1 stage without persistent inactivation of p16/Rb pathway, p53 pathway, and the unknown pathway involving 1p34 locus. The importance of the p16/Rb and p53 pathways in lung epithelial cells is known by the high incidence of their defects in any histological type of lung cancer (25), and the adenocarcinomas without their persistent inactivation may have come without these checkpoints.

It has been indicated that only a small proportion of the tumor cells are able to form colonies in an *in vitro* colonogenic assay (26), and we found that adenocarcinoma cell lines without persistent inactivation of p16/Rb pathway had higher colony formation capacity in soft agar. Moreover, CDDP-resistant clone with intact p16/Rb (PC-9/CDDP), but not one with inactivation (PC-14/CDDP), showed higher colony formation capacity than the parent clones. Although the evidence is not sufficient yet, this could be explained that the former contained CSCs and enriched them in the CDDP-resistant clone, because CSCs are considered to be resistant to conventional chemotherapeutic drugs (2), while the latter did not contain CSCs originally and could not enrich the resistant clone. We also examined the expression levels of the known cancer stem cell markers, *PROM1* (2), *BMI1* (7), and *ABCG2* (27) in 15 lung cancer cell lines, and found that the p16/Rb intact cell line RERF-LC-Ad2 showed markedly high expression of *PROM1*, supporting our hypothesis that the p16/Rb intact immortal cancer cells may have derived from stem cells, and none of the presently available CSC markers is sufficient for all types of lung cancer.

Taken together, the present data indicate that 7 (50%) of 14 lung adenocarcinoma tissues with high telomerase activity (indicating they are predominantly consist of immortal cancer cells) and 3 of 11 (or 2 of 9 parent) lung adenocarcinoma cell lines, PC-9, PC-9/CDDP, and RERF-LC-Ad2, may have developed from M1 escaped cells, i.e., innately telomerase-positive stem cells, which are considered to be the origin of CSCs. Some of SCLC may have derived from such cells, because 1 of 6 SCLC tissues with high telomerase activity and 1 original (PC-6) as well as its 2 drug-resistant cell clones showed intact p16/Rb pathway (Fig. 7). However, for squamous cell carcinoma, no tissue with high telomerase activity nor the cell line examined showed intact p16/Rb pathway, indicating that it is always required to overcome the M1 stage to become immortal squamous cell carcinoma cells and that the telomerase-negative somatic cells may be of such origin. This speculation may be partly supported by the facts that squamous cell carcinoma often demonstrates evidence of multistep carcinogenesis, i.e., hyperplasia, metaplasia, dysplasia, carcinoma *in situ*, and invasive cancer, while adenocarcinoma and SCLC often lack such precancerous lesions indicating *de novo* carcinogenesis. Thus, we propose that there are two distinct pathways in lung carcinogenesis: one from primarily telomerase-positive cells, i.e., stem cells, independent of p16/Rb checkpoint mechanism in M1 stage, while the other from telomerase-negative somatic cells overcoming the M1 stage by inactivation of the p16/Rb and p53 pathways.

In conclusion, our study showed new important insights into lung CSCs. In particular, high telomerase expression without p16/Rb aberration possibly is a marker of cancer stem cells in lung cancers. Although further experiments are necessary to confirm our hypothesis, it could be an important key to study the molecular mechanisms of lung carcinogenesis and clinical implications.

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## References


1. Clarke MF, Dick JE, Dirks PB, Eaves CJ, Jamieson CH, Jones DL, Visvader J, Weissman IL and Wahl GM: Cancer stem cells - perspectives on current status and future directions: AACR Workshop on cancer stem cells. Cancer Res 66: 9339-9344, 2006.
2. Eramo A, Lotti F, Sette G, Pilozzi E, Biffoni M, Di Virgilio A, Conticello C, Ruco L, Peschle C and De Maria R: Identification and expansion of the tumorigenic lung cancer stem cell population. Cell Death Differ 15: 504-514, 2008.
3. Mizrak D, Brittan M and Alison MR: CD133: molecule of the moment. J Pathol 214: 3-9, 2008.
4. Hirschmann-Jax C, Foster AE, Wulf GG, Nuchtern JG, Jax TW, Gobel U, Goodell MA and Brenner MK: A distinct 'side population' of cells with high drug efflux capacity in human tumor cells. Proc Natl Acad Sci USA 101: 14228-14233, 2004.
5. Jiang F, Qiu Q, Khanna A, Todd NW, Deepak J, Xing L, Wang H, Liu Z, Su Y, Stass SA and Katz RL: Aldehyde dehydrogenase 1 is a tumor stem cell-associated marker in lung cancer. Mol Cancer Res 7: 330-338, 2009.
6. Meng X, Li M, Wang X, Wang Y and Ma D: Both $CD133^+$ and $CD133^-$ subpopulations of A549 and H446 cells contain cancer-initiating cells. Cancer Sci 100: 1040-1046, 2009.

7. Lobo NA, Shimono Y, Qian D and Clarke MF: The biology of cancer stem cells. Annu Rev Cell Dev Biol 23: 675-699, 2007.
8. Hiyama E and Hiyama K: Telomere and telomerase in stem cells. Br J Cancer 96: 1020-1024, 2007.
9. Hiyama K, Hiyama E, Tanimoto K and Nishiyama M: Role of telomeres and telomerase in cancer. In: Telomeres and Telomerase in Cancer. Hiyama K (ed.). Humana Press, Springer, New York, NY, pp171-180, 2009.
10. Shapiro GI, Edwards CD, Ewen ME and Rollins BJ: $p16^{INK4A}$ participates in a G1 arrest checkpoint in response to DNA damage. Mol Cell Biol 18: 378-387, 1998.
11. Shay JW and Wright WE: Senescence and immortalization: role of telomeres and telomerase. Carcinogenesis 26: 867-874, 2005.
12. Arifin M, Hiyama K, Tanimoto K, Wiyono WH, Hiyama E and Nishiyama M: EGFR activating aberration occurs independently of other genetic aberrations or telomerase activation in adenocarcinoma of the lung. Oncol Rep 17: 1405-1411, 2007.
13. Sobin LH, Hermanek P and Hutter RV: TNM classification of malignant tumors. A comparison between the new (1987) and the old editions. Cancer 61: 2310-2314, 1988.
14. Hiyama K, Hiyama E, Ishioka S, Yamakido M, Inai K, Gazdar AF, Piatyszek MA and Shay JW: Telomerase activity in small-cell and non-small-cell lung cancers. J Natl Cancer Inst 87: 895-902, 1995.
15. Hiyama K, Ishioka S, Shirotani Y, Inai K, Hiyama E, Murakami I, Isobe T, Inamizu T and Yamakido M: Alterations in telomeric repeat length in lung cancer are associated with loss of heterozygosity in p53 and Rb. Oncogene 10: 937-944, 1995.
16. Mendoza C, Sato H, Hiyama K, Ishioka S, Isobe T, Maeda H, Hiyama E, Inai K and Yamakido M: Allelotype and loss of heterozygosity around the L-myc gene locus in primary lung cancers. Lung Cancer 28: 117-125, 2000.
17. Tanaka T, Tanimoto K, Otani K, Satoh K, Ohtaki M, Yoshida K, Toge T, Yahata H, Tanaka S, Chayama K, Okazaki Y, Hayashizaki Y, Hiyama K and Nishiyama M: Concise prediction models of anticancer efficacy of 8 drugs using expression data from 12 selected genes. Int J Cancer 111: 617-626, 2004.
18. Hiyama K, Tanimoto K, Nishimura Y, Tsugane M, Fukuba I, Sotomaru Y, Hiyama E and Nishiyama M: Exploration of the genes responsible for unlimited proliferation of immortalized lung fibroblasts. Exp Lung Res 34: 373-390, 2008.
19. Herman JG, Graff JR, Myohanen S, Nelkin BD and Baylin SB: Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci USA 93: 9821-9826, 1996.
20. Hiyama K, Hiyama E and Shay JW: Telomeres and telomerase in humans. In: Telomeres and Telomerase in Cancer. Hiyama K (ed.). Humana Press, Springer, New York, NY, pp3-21, 2009.
21. Hiyama E, Hiyama K, Yokoyama T and Shay JW: Immunohistochemical detection of telomerase (hTERT) protein in human cancer tissues and a subset of cells in normal tissues. Neoplasia 3: 17-26, 2001.
22. Kumazaki T, Hiyama K, Takahashi T, Omatsu H, Tanimoto K, Noguchi T, Hiyama E, Mitsui Y and Nishiyama M: Differential gene expressions during immortalization of normal human fibroblasts and endothelial cells transfected with human telomerase reverse transcriptase gene. Int J Oncol 24: 1435-1442, 2004.
23. Miyazu YM, Miyazawa T, Hiyama K, Kurimoto N, Iwamoto Y, Matsuura H, Kanoh K, Kohno N, Nishiyama M and Hiyama E: Telomerase expression in non-cancerous bronchial epithelia is a possible marker of early development of lung cancer. Cancer Res 65: 9623-9627, 2005.
24. Kraunz KS, Nelson HH, Lemos M, Godleski JJ, Wiencke JK and Kelsey KT: Homozygous deletion of $p16^{INK4a}$ and tobacco carcinogen exposure in nonsmall cell lung cancer. Int J Cancer 118: 1364-1369, 2006.
25. Yokota J and Kohno T: Molecular footprints of human lung cancer progression. Cancer Sci 95: 197-204, 2004.
26. Heppner GH: Tumor heterogeneity. Cancer Res 44: 2259-2265, 1984.
27. Lou H and Dean M: Targeted therapy for cancer stem cells: the patched pathway and ABC transporters. Oncogene 26: 1357-1360, 2007.