Persistent Increase in Chromosome Instability in Lung Cancer

Possible Indirect Involvement of p53 Inactivation

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Karyotype and fluorescence *in situ* hybridization analyses have demonstrated the frequent presence of an altered static state of the number of chromosomes (ie, aneuploidy) in lung cancer, but it has not been directly established whether aneuploidy is in fact associated with a persistent increase in the rate of chromosomal losses and gains (ie, chromosome instability, or CIN). The study presented here used a panel of 10 lung cancer cell lines to provide for the first time direct evidence that CIN is a common feature in lung cancer cell lines in association with the presence of significant aneuploidy. In addition, we found that the CIN phenotype correlates well with the presence of p53 mutations. However, human papilloma virus 16-E6-directed inactivation of p53 in a representative non-CIN lung cancer cell line did not result in the induction of CIN, at least up to the 25th generation, suggesting that inactivation of p53 itself is unlikely to directly induce CIN in lung cancer cells. Interestingly, however, significant CIN could be induced in conjunction with the generation of aneuploid populations when the mitotic spindle formation was transiently abrogated in p53-inactivated cells. These results suggest that inactivation of p53 may allow lung cancer cells to go through an inappropriate second division cycle under certain forms of mitotic stresses, which would result in the induction of the CIN phenotype in conjunction with the generation of aneuploidy. (Am J Pathol 2001, 159:1345–1352)

Lung cancer currently claims more than 160,000 lives annually as the number one cause of cancer death in the

United States, and it has also become the leading cause in Japan with more than 47,000 deaths annually.¹ Lung cancer cells have been shown to carry frequent structural chromosomal abnormalities such as deletions, which presumably reflect genetic lesions at tumor suppressor loci.^{2,3} In addition, previous cytogenetic studies have shown that lung cancer cells frequently exhibit numerical changes in chromosomes.⁴ It is conceivable that this form of chromosomal abnormalities may contribute to tumor development and progression by facilitating loss of heterozygosity and the phenotypic manifestation of inactivated tumor suppressor genes, as well as favoring polysomy of chromosomes that harbor growth-promoting genes.⁵

Karyotype and fluorescence *in situ* hybridization (FISH) analyses have demonstrated the frequent occurrence of altered static state of the number of chromosomes (ie, aneuploidy) in lung cancer, but it has not been directly established whether aneuploidy is indeed associated with a persistent increase in the rate of chromosomal losses and gains (ie, chromosome instability, or CIN), or whether it is merely a fingerprint of a few chaotic, non-persisting chromosomal missegregations. In addition, very few genetic and biochemical data are available at present about how this very common abnormality is acquired in lung cancers. In this connection, Lengauer and his colleagues⁶ recently showed that human colon cancer cell lines frequently displayed a CIN phenotype. They also found a consistent association of CIN in colon cancers with dysfunction of the mitotic checkpoint as well as with infrequent mutations in two mitotic checkpoint genes, *BUB1* and *BUBR1*.⁷ As for lung cancer, we previously reported that the mitotic checkpoint is impaired in ~40% of lung cancer cell lines and that the *MAD1* mitotic checkpoint gene is infrequently altered in lung cancer,

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whereas other mitotic checkpoint genes, including *MAD2*, *BUB1*, *BUBR1*, and *BUB3*, did not show any evidence of mutations in lung cancer.^{8–10}

The study presented here demonstrates that the CIN phenotype is indeed persistently present in lung cancer cell lines and shows a good concordance with the presence of significant aneuploidy. In addition, our data show that the CIN phenotype correlates well with the presence of *p53* mutations. Interestingly, however, inactivation of the *p53* function by human papilloma virus (HPV)16-E6 infection did not readily induce the CIN phenotype in a chromosomally stable lung cancer cell line, but rather seemed to permit propagation of aneuploid cells accompanying the CIN phenotype under certain forms of mitotic stresses.

Materials and Methods

Cell Lines

Cells were cultured in RPMI 1640 medium supplemented with 5% fetal calf serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. The cell lines ACC-LC-319, ACC-LC-94, and ACC-LC-176 were established at our laboratory. NCI-H460 and HCT116 were obtained from the American Type Culture Collection (Manassas, VA). Other cell lines were generously provided by Drs. L. J. Old and M. Akiyama (Memorial Sloan-Kettering Cancer Center and Radiation Effect Research Foundation, respectively).

FISH for Detection of Chromosome Instability

Chromosome instability was examined essentially with the methods developed by Lengauer and colleagues.⁶ In brief, cells were transfected with pcDNA3 using DMRIE-C (Life Technologies, Inc., Rockville, MD), followed by selection with G418. FISH analysis of methanol/acetic acid (3:1)-fixed nuclei of multiple single-cell-derived clones was performed by using centromeric probes specific for chromosomes 1, 11, 12, and 17 at the 15th and 25th generations after transfection. At least 100 nuclei were evaluated per clone with each chromosome probe. In addition, chromosomal variation *in situ* was evaluated by means of FISH on small colonies consisting of 100 to 200 cells, which had been grown directly on microscopic slides and fixed with 100% ethanol, essentially in the manner described by Lengauer and colleagues.⁶

Centrosome Staining

Exponentially growing cells were plated at a density of 5×10^4 cells/well on 24-mm glass coverslips in 12-well plates. After a 48-hour incubation in RPMI 1640 containing 5% fetal calf serum, the cells were washed with phosphate-buffered saline (PBS), sequentially fixed in ice-cold methanol/acetone, preincubated with Tris-buffered saline (0.9% NaCl in 50 mmol/L Tris-HCl, pH 8.0) containing 1% bovine serum albumin and 1% skim milk for 1 hour, and then subjected to incubation with a polyclonal antibody to pericentrin (BAbCO, Richmond, CA) at

a dilution of 1:150 for 1 hour. After extensive washing, the slides were incubated with a fluorescein isothiocyanate-conjugated secondary antibody, and examined with a fluorescence microscope after counterstaining with 4', 6-diamidino-2-phenylindole. To determine centrosome abnormalities in each cell line, the diameter and number of pericentrin spots of at least 100 cells were carefully examined. Centrosomes were considered to be abnormal if they were more than three times larger in diameter than human normal fibroblasts and/or if there were three or more centrosomes per cell. Abnormalities in both numbers and size abnormalities were evaluated together, because it was occasionally difficult to clearly distinguish between one large centrosome and an aggregate of multiple centrosomes under conventional fluorescent microscopy. The criteria used were: ++, >10% of cells had abnormal centrosomes; +, 3 to 10% of cells showed abnormal staining; –, 0 to <3% of cells had abnormal centrosomes. The frequency of abnormal staining in human normal fibroblasts was <3%.

Northern Blot Analysis of *STK15/BTAK/aurora2*

Northern blot analysis was performed following the standard procedures. A 744-bp cDNA probe of the *STK15/BTAK/aurora2* gene was generated by polymerase chain reaction (PCR) amplification with the aid of 5'-AAGCT-CAGCTGGAGAAAGCC and 5'-CTCAAGGATTCTC-CCCCTG oligonucleotide primes. Induction of G₁ phase arrest in ACC-LC-48 was performed, as described previously.¹¹

Analysis of Mitotic Delay in Response to Nocodazole

Cells were cultured in the presence of nocodazole, harvested at 12, 18, 24, 32, 40, and 52 hours, and then fixed with 4% formaldehyde in PBS, followed by staining with 4', 6-diamidino-2-phenylindole. Two hundred nmol/L of nocodazole was used for all cell lines except for QG56, to which 800 nmol/L of nocodazole was added. To measure the mitotic index (percentage of viable cells arrested in mitosis), fluorescence microscopy was used to count at least 300 cells for each measurement as described previously,⁸ which was repeated at least twice. Two control cell lines with known mitotic checkpoint defects (SW480 and T47D)⁷ were also included. Flow cytometric analysis was also conducted to evaluate the cell-cycle profile of cells harvested at various time points before and after 32 hours incubation with nocodazole. Cells were stained with propidium iodide and analyzed with the aid of FAC-Scan and Cell Fit-DNA software (Becton Dickinson, Bedford, MA).⁸

Detection of *p53* Mutations and Functional Inactivation of *p53* by HPV16-E6 Infection

p53 mutations in each cell line were examined by sequencing as described previously.¹² Functional inactivation

Table 1. Variations in Numbers of Chromosomes at 15th Generation in Clones Generated from a Single Cell of Human Lung Cancer Cell Lines

Cell line	Number of chromosomes	Clone	% with losses or gains				Instability score*
			Ch. 1	Ch. 11	Ch. 12	Ch. 17	
QG90	84	a	36% (4) [†]	33% (4)	29% (4)	33% (4)	1.0 (16/16)
		b	27% (4)	20% (4)	26% (4)	32% (4)	
		c	41% (4)	33% (4)	31% (4)	32% (4)	
		d	40% (4)	25% (4)	35% (4)	46% (4)	
PC-10	102	a	32% (5)	17% (3)	30% (5)	37% (4)	1.0 (16/16)
		b	39% (4)	61% (3)	43% (5)	47% (4)	
		c	61% (4)	40% (3)	63% (4)	54% (4)	
		d	55% (5)	41% (3)	48% (5)	57% (3)	
QG56	69	a	22% (3)	21% (3)	26% (3)	31% (3)	1.0 (12/12)
		b	28% (3)	23% (3)	26% (3)	27% (4)	
		c	18% (3)	13% (3)	19% (2)	23% (4)	
Calu1	87	a	53% (4)	18% (3)	63% (7)	63% (5)	1.0 (8/8)
		b	66% (4)	53% (3)	81% (6)	73% (5)	
SK-MES-1	86	a	16% (4)	16% (3)	50% (3)	44% (2)	1.0 (8/8)
		b	19% (4)	16% (4)	46% (3)	40% (2)	
ACC-LC-94	50	a	13% (2)	26% (3)	30% (4)	12% (2)	0.88 (7/8)
		b	8% (2)	18% (3)	58% (4)	27% (3)	
NCI-H460	57	a	10% (2)	1% (2)	12% (2)	15% (2)	0.67 (8/12)
		b	33% (4)	3% (2)	12% (2)	19% (2)	
		c	20% (4)	4% (2)	16% (2)	17% (2)	
ACC-LC-319	70	a	34% (4)	9% (3)	17% (4)	9% (2)	0.63 (5/8)
		b	35% (4)	11% (2)	17% (4)	8% (2)	
PC-1	48	a	9% (2)	5% (2)	4% (2)	4% (2)	0.33 (4/12)
		b	11% (2)	13% (2)	7% (2)	8% (2)	
		c	14% (2)	8% (2)	16% (2)	9% (2)	
ACC-LC-176	44	a	6% (2)	8% (2)	6% (2)	4% (2)	0 (0/16)
		b	8% (2)	8% (2)	6% (2)	9% (2)	
		c	6% (2)	10% (2)	3% (2)	10% (2)	
		d	5% (2)	4% (2)	3% (2)	5% (2)	
HCT116	45	a	7% (2)	6% (2)	4% (2)	7% (2)	0 (0/8)
		b	9% (2)	9% (2)	3% (2)	7% (2)	

*(Number of observations of either loss or gain of chromosomes in more than 10% of cells)/(chromosomes × clones examined in each cell line).

[†]Number in parentheses shows number of modal chromosomes in each clone.

tion of p53 in ACC-LC-176 was accomplished by infecting a replication-defective retrovirus vector (LXSN) carrying the HPV16-E6 gene, followed by selection with G418, as described previously.¹³ Infected cells were irradiated with 12-Gy X-ray, lysed in Laemmli's sample buffer, and examined for induction of the p53 protein by Western blot analysis using an anti-p53 monoclonal antibody (Ab6) (Oncogene Research Products, Cambridge, MA).

Results

Detection of CIN in Lung Cancer Cell Lines

Ten lung cancer cell lines were examined for the presence of persistent changes in the number of chromosomes by FISH analysis of interphase cells with the aid of four centromeric probes. Clones of each of the 10 cell lines were generated and chromosome losses or gains in lung cancer cells within a defined period of time were examined by FISH at the 15th and 25th generations.

As a result, we could divide the 10 cell lines into three groups, similar results being obtained at the 15th and 25th generations for each of the cell lines (Table 1, data not shown for the 25th generation). The first group consisted of five cell lines, QG90, QG56, PC-10, Calu1, and

SK-MES-1, which showed marked variation in the number of chromosomes within each of the multiple single-cell-derived clones. For example, the QG90 clone showed that only 64% of cells had a modal number of chromosome 1 (ie, presumably the original number of chromosome 1 in this clone), whereas 24 and 12% of cells showed losses and gains, respectively. FISH analysis using probes specific for chromosomes 11, 12, and 17 similarly revealed dramatic variations in chromosome numbers. Evaluation of the three other clones of QG90 confirmed the reproducibility of these observations. In our study we used an arbitrary instability score, which was based on the following calculation: (number of observations, in which >10% of the cells had other than a modal number of chromosomes)/(chromosomes × clones examined in each cell line). According to this score, all of the five cell lines belonging to this group had an instability score of 1.0 and the rate of CIN could be estimated to be in excess of 10^{-2} per generation for each chromosome analyzed. These cell lines without exception were found to be markedly aneuploid. In contrast, a near-diploid lung cancer cell line ACC-LC-176 as well as a colon cancer cell line HCT116, which was included as a non-CIN control, were found to have acquired chromosomal losses or gains in <10% of cells in all clones and chromosomes examined, for an instability score of 0. The

Table 2. Abnormalities in Mitotic Delay, Centrosome, *STK15/BTAK/aurora2*, and *p53* Status in Relation to the Presence of CIN

CIN phenotype*	Cell line	Mitotic delay†	Centrosome abnormality‡	<i>STK15/BTAK/aurora2</i> §	<i>p53</i> Gene status
Unstable	QG90	Normal	+	++	Pro to Ser (codon 249)
	PC-10	Abnormal	++	++	Gly to Cys (codon 245)
	QG56	Normal	—	++	Pro to Ser (codon 249)
	Calu1	Abnormal	++	++	Homozygous deletion
	SK-MES-1	Normal	++	++	Glu to Stop (codon 298)
Intermediate	ACC-LC-94	Normal	+	++	Asp to Gly (codon 281)
	NCI-H460	Normal	—	++	Wild-type
	ACC-LC-319	Abnormal	++	++	1-bp deletion (codon 71)
	PC-1	Abnormal	—	++	Wild-type
Stable	ACC-LC-176	Normal	—	++	Wild-type
Control	HCT116	Normal	—	N.D.	Wild-type

*Unstable, instability score 1.0; intermediate, instability scores between 0 and 1.0; stable, 0.

†Data for QG90, PC-10, QG56, NCI-H460, ACC-LC-319, PC-1, and ACC-LC-176 from our previous study (Takahashi et al⁸) were used.

‡Centrosomes were considered to be abnormal if they were more than three times larger in diameter than human normal fibroblasts and/or if there were three or more centrosomes per cell. ++, >10% of cells had abnormal centrosomes; +, 3 to 10% of cells showed abnormal staining, —, 0 to <3% of cells had abnormal centrosomes. The frequency of abnormal staining in human normal fibroblasts was less than 3% both in number and size in this assay.

§*STK15/BTAK/aurora2* expression was classified as follows. ++, significantly higher expression than that in normal lung; +, expression comparable to that in normal lung; —, no expression.

remaining group, ACC-LC-94, NCI-H460, ACC-LC-319, and PC-1, showed an intermediate phenotype with considerable variation in the instability score, ranging from 0.33 to 0.88.

Chromosomal variations *in situ* were also examined by analyzing small colonies consisting of only 100 to 200 cells, as described by Lengauer and colleagues.⁶ Cell lines were plated at low density on microscope slides so that independent clones could be examined after seven to eight generations by FISH without disaggregating them. Examination of five independent colonies of PC-10 with the unstable phenotype showed that 52% (range, 47 to 57%) and 54% (range, 49 to 60%) of the cells had acquired either losses or gains in comparison with the modal numbers of chromosomes 11 and 12, respectively (data not shown for individual colonies). These results were thus consistent with the presence of high rates of change in chromosome numbers. In contrast, ACC-LC-176 with the stable phenotype exhibited little chromosomal variation within each of the three independent colonies, with only 6% (range, 5 to 7%) of the cells having other configurations than two chromosomes 11.

Presence of Mitotic Checkpoint Defects in a Fraction of Cell Lines with CIN

The mitotic checkpoint is thought to be essential for ensuring accurate chromosome segregation by implementing mitotic delay in response to a spindle defect.^{14,15} The panel of lung cancer cell lines used in our study was therefore examined for the presence of mitotic checkpoint impairment in relation to the presence of CIN (Table 2). Two cell lines each of the unstable and intermediate phenotype groups showed a significant reduction (mean, 21%; range, 17 to 25%) of cells arrested at mitosis. This reduction was comparable to the mitotic indices of the control cell lines (SW480 and T47D) with known mitotic checkpoint defects (25 and 21%, respectively),⁷ when treated with a microtubule poison, nocodazole. In con-

trast, the stable lung cancer cell line ACC-LC-176 and the non-CIN control HCT116 exhibited normal responses (peak mitotic indices, 48 and 57%, respectively).

Association of CIN with the Presence of Centrosome Abnormalities

It has been suggested that abnormality in centrosomes, which play a vital role in organizing mitotic spindle during cell division, may be involved in the acquisition of CIN.^{16,17} The panel of lung cancer cell lines was used to study centrosome abnormalities in relation to CIN by using an antibody against pericentrin, a component of the centrosome matrix protein involved in microtubule nucleation (Table 2 and Figure 1).¹⁸ Four of the five unstable cell lines (80%) exhibited abnormal centrosome staining (mean, 13%; range, 4 to 21%), whereas three cell lines showed abnormality in >10% of the cells with a frequency of up to 21%. Two of the four cell lines with an intermediate phenotype also showed abnormal centrosome staining. Both ACC-LC-176 and HCT116 without CIN exhibited abnormal centrosome staining in <3% of the cells.

Ubiquitous High Expression Levels of *STK15/BTAK/aurora2*

Northern blot analysis was performed to examine the expression of *STK15/BTAK/aurora2* centrosome kinase. All lung cancer cell lines examined showed significantly higher expression levels than normal lung tissues, regardless of the presence or absence of CIN (Figure 2). Interestingly, a high *STK15/BTAK/aurora2* expression level was also detectable in normal lung epithelial cell lines, whereas G₁-arrested lung cancer cell lines expressed it at a very low level, comparable to that in normal lung tissues.

Figure 1. Representative abnormal centrosome staining in SK-MES-1 with significant CIN. Centrosomes were considered abnormal if there were three or more signals and/or the diameter was more than three times larger than that of human normal fibroblasts. Representative SK-MES-1 cells exhibiting normal centrosome staining (arrow in A, enlarged figure in B) and abnormalities in size (open arrowhead in A, enlarged figure in C) or numbers (solid arrowhead in A, enlarged figure in D). Representative centrosome staining of normal fibroblasts is shown at the same magnification in the inset of B.

Association of CIN with the Presence of *p53* Mutations

It has been reported that embryonic fibroblasts of *p53* knockout mouse exhibit mitotic checkpoint impairment and centrosome abnormality, leading to an increase in CIN.^{19,20} All of the five cell lines with marked CIN as well as two of the four cell lines with an intermediate phenotype were found to carry *p53* mutations. Notably, all cell lines with centrosome abnormalities carried *p53* alterations including both missense and null mutations. On the other hand, all but one cell line with mutant *p53* exhibited centrosome abnormalities. Three of the four cell lines with impaired response to nocodazole carried altered *p53*.

Figure 2. Ubiquitous high expression levels of STK15/BTAK/aurora2 in lung cancer cell lines. Note that the G₁-arrested ACC-LC-48 lung cancer cell line shows a low STK15/BTAK/aurora2 expression level comparable to that in normal lung tissues.

Figure 3. Functional *p53* inactivation by HPV-E6 infection in ACC-LC-176. **A:** Western blot analysis of *p53* in HPV16-E6-infected clones. Induction of *p53* in response to irradiation, which is clearly seen in the control clone (La), is abrogated in HPV16-E6-infected clones (Ea, Eb, and Ed). **B:** Mitotic indices of HPV16-E6-infected clones of ACC-LC-176 in the presence of nocodazole. Transient accumulation of cells at the metaphase and subsequent adaptation is seen within 32 hours regardless of the *p53* status.

Indirect Role of *p53* Inactivation in the Induction of Aneuploid Cells with CIN

We also investigated whether functional inactivation of *p53* can directly induce CIN in association with mitotic checkpoint impairment and/or centrosome abnormality. A non-CIN lung cancer cell line ACC-LC-176 was infected with a retrovirus carrying HPV16-E6. Abrogation of *p53* induction in response to irradiation or nocodazole treatment was confirmed by Western blot analysis of HPV16-E6-infected clones (Figure 3A, data not shown for nocodazole). FISH analysis of five independent HPV16-E6-infected clones at both the 15th and 25th generation revealed that *p53* inactivation by itself did not appreciably induce CIN, mitotic checkpoint impairment or centrosome abnormality (Figure 3B and Table 3; data not shown for the 15th generation).

Because chemotherapeutic agents affecting the mitotic spindle, such as vinorelbine, have been introduced to clinical practice, we here investigated whether transient abrogation of mitotic spindle formation, a condition

Table 3. Lack of Direct Induction of CIN by HPV16-E6-Directed Inactivation of p53 in ACC-LC-176 Cells

Infection	Clone	% with losses or gains		Instability score*	Mitotic delay	Centrosome abnormality [†]
		Ch. 11	Ch. 12			
LXSN	La	6% (2) [‡]	5% (2)	0 (0/10)	Normal	—
	Lb	5% (2)	1% (2)		Normal	—
	Lc	8% (2)	8% (2)		N.D.	—
	Ld	5% (2)	9% (2)		N.D.	—
	Le	6% (2)	8% (2)		N.D.	—
HPV16-E6	Ea	6% (2)	3% (2)	0.1 (1/10)	N.D.	—
	Eb	5% (2)	1% (2)		Normal	—
	Ec	2% (2)	5% (2)		N.D.	—
	Ed	12% (2)	7% (2)		Normal	—
	Ee	4% (2)	7% (2)		N.D.	—

*Fraction of events (i.e., losses or gains compared with modal number of chromosomes) observed in the two infections.

[†]Determined as described in the footnote of Table 2.

[‡]Numbers in parentheses show modal numbers of each chromosome.

mimicking chemotherapy, may lead to the generation of aneuploid cells and whether such cells exhibit CIN. Two HPV16-E6-infected clones, Eb and Ed, as well as a control clone, La, were incubated with nocodazole for 32 hours, followed by thorough washing with fresh medium to remove the agent. A small fraction of the treated cells, which had survived the treatment, could subsequently be propagated (Figure 3B). Fluorescence-activated cell sorting analysis at various time points up to 12 days revealed that, after the transient nocodazole treatment, cells with a content of >2N DNA could survive in p53-inactivated clones (Eb and Ed), whereas predominance

of cells with a content of 2N DNA gradually became apparent in the surviving control clone (La) (Figure 4). Consistent with this finding, FISH analysis using a probe specific for chromosome 11 showed that, in contrast to the predominance of diploid colonies in clone La, the vast majority of the colonies of clones Eb and Ed were aneuploid. Interestingly, these colonies were found to exhibit marked CIN during the analysis of chromosomal variations *in situ*, resulting in either losses or gains of chromosome 11 in 33% (range, 24 to 44%) of the cells in clone Eb and in 36% (range, 27 to 44%) of those in clone Ed.

Figure 4. FACS and FISH analyses after transient nocodazole treatment of HPV16-E6-infected ACC-LC-176 clones. Preferential propagation of aneuploid populations was observed in p53-inactivated clones (Eb and Ed) but not in a clone with normal p53 (La).

Discussion

In the study presented here, we have provided for the first time direct evidence that CIN is a common feature in lung cancer cell lines in consistent association with the presence of significant aneuploidy and *p53* mutations. However, our results also indicate that it is unlikely that inactivation of *p53* directly induces CIN in lung cancer cells, because HPV16-E6-directed inactivation of *p53* in a representative non-CIN lung cancer cell line did not result in the induction of CIN at least up to the 25th generation. Importantly, however, significant CIN could be induced in conjunction with the generation of aneuploid populations, when mitotic spindle was transiently eliminated in *p53*-inactivated cells. Although it has been shown that *p53* prohibits subsequent endoreduplication in the prolonged presence of nocodazole,^{21–23} our study suggests that *p53* may play a role in the imposition of sustained arrest in a G_1 -like state associated with 4N DNA content in response to transient disruption of mitotic spindles. Thus, inactivation of *p53* seems to allow lung cancer cells to go through an inappropriate second division cycle under certain types of mitotic stresses, resulting in the induction of the CIN phenotype by means of a presently unclear mechanism. In addition to artificial disruption of mitotic spindles by nocodazole, mitotic spindle-affecting chemotherapeutic agents as well as alterations in the mitotic apparatus and checkpoint might also have similar consequences, leading to the generation of aneuploid populations with CIN in lung cancers carrying mutant *p53*. In fact, transient treatment with 5 nmol/L vinorelbine, which is an active agent for the treatment of lung cancer, similarly induced an aneuploid population in the *p53*-inactivated clones (Eb and Ed) but not in clone La with the wild-type *p53* function (data not shown). It is possible that induction of aneuploid populations may in turn increase the chances for surviving lung cancer cells to acquire selective growth advantage after chemotherapy, as it has been suggested that CIN may contribute to tumor heterogeneity and progression.⁵

It is conceivable that once CIN is acquired, lung cancers are increasingly likely to generate karyotypes altered to most suitable for genetic lesions, which are distinct in each case. Selective growth advantage may be acquired by the loss of the wild-type allele of tumor suppressor genes as a result of loss of heterozygosity and an increase in the gene dosage of growth-promoting genes by polysomy as well as by changes in the expression profiles of thousands of genes on the affected chromosomes. In this connection, the presence of allele-specific imbalance in various chromosomes in lung cancer, which has been reported by us and others,^{24,25} might also reflect such a CIN-directed selection of chromosomes containing genes with allele-specific expression.

We note that our results are markedly different from those for colon cancer, because it has been reported that cell fusions of two non-CIN colon cancer cell lines,⁶ hence artificially creating aneuploid cells, did not result in the induction of CIN. It is possible that fundamental differences in the ways aneuploid cells were created (ie, transient nocodazole treatment *versus* cell fusion) or dis-

tinct genetic backgrounds of the cell lines used might account for this difference. CIN in lung cancer appears to be less closely related to the presence of mitotic checkpoint impairment than that in colon cancer, because all colon cancer cell lines with a CIN phenotype reportedly had mitotic checkpoint impairment.⁷ This finding also points to the possibility that cancer type-specific differences may exist in the major underlying causes of CIN of these two common cancers of adults. Our results, however, do not preclude a role for mitotic checkpoint impairment in the acquisition of CIN in lung cancer. In fact, we previously reported that the *MAD1* mitotic checkpoint gene is indeed mutated in lung cancer *in vivo*, although with low frequency.⁹

Although there is increasing evidence of a direct link between centrosome dysfunction and chromosome mis-segregation during tumorigenesis,^{26,27} inactivation of *p53* has been reported to induce abnormal centrosome amplification, resulting in unequal segregation of chromosomes.¹⁹ Although the present study suggests a potential connection between the presence of centrosome abnormalities and *p53* mutations in the acquisition of CIN, we did not observe significant abnormalities in the numbers or size of centrosomes in *p53*-inactivated clones and identical *p53* mutation was present in two cell lines, one with and the other without centrosome abnormalities. Exogenous overexpression of a human serine/threonine kinase *STK15/BTAK/aurora2* was previously claimed to cause abnormal centrosome amplification, leading to aneuploidy and transformation,²⁸ but the ubiquitous high expression levels observed in our study, regardless of the presence or absence of CIN, suggests that the functional significance of a high *STK15/BTAK/aurora2* expression level needs further clarification in terms of the induction of CIN in lung cancer cells.

As to possible other mechanisms that might make lung cancer cells susceptible to the acquisition of CIN, there is a large number of potential targets, alterations of which can theoretically give rise to CIN. These include genes involved in chromosome condensation, cohesion, kinetochore structure and function, centrosome/microtubule formation, and various checkpoints such as cytokinesis checkpoint.⁵ In this connection, our preliminary examination of 39 lung cancer cell lines, including all 10 cell lines examined here, did not find any alterations in the human *EB1* gene, a yeast homologue of which has been implicated in cytokinesis checkpoint,²⁹ whereas 44 primary lung cancer specimens did not carry any mutations in the human *securin* gene (unpublished observation), which is thought to play a role in sister chromatid separation.³⁰

Evidence to date indicates that further studies are needed to identify and clarify the underlying mechanisms directly responsible for the induction of CIN in lung cancer. In this regard, human homologues, which, when altered, can result in CIN phenotypes in yeast, would be excellent candidates for such genetic lesions. Clarification of the molecular bases and biological significance of CIN should provide important clues for the development of new therapeutic approaches for this fatal cancer.

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