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Identification of Frequent G_2 Checkpoint Impairment and a Homozygous Deletion of *14-3-3ϵ* at 17p13.3 in Small Cell Lung Cancers[1]

Hiroyuki Konishi, Taku Nakagawa, Tomoko Harano, Kotaro Mizuno, Hiroko Saito, Akira Masuda, Hikaru Matsuda, Hirotaka Osada, and Takashi Takahashi[2]

Division of Molecular Oncology, Aichi Cancer Center Research Institute, Kanokoden, Chikusa-ku, Nagoya 464-8681 [H. K., T. N., T. H., K. M., H. S., A. M., H. O., T. T.], and Department of Surgery, Osaka University Graduate School of Medicine, Yamadaoka, Suita 565-0871 [H. K., H. M.], Japan

ABSTRACT

Accumulating evidence suggests that a coordinately controlled G_2 checkpoint prevents cells with damaged DNA from entering mitosis, thus playing an important role in the maintenance of chromosomal integrity. In the study presented here, we identified a homozygous deletion of the *14-3-3ϵ* gene, which resides within a previously identified, commonly deleted region at 17p13.3 in lung cancers, in two small cell lung cancer cell lines that originate from distinct metastatic sites of the same patients. The introduction of *14-3-3ϵ* induced significantly restored G_2 checkpoint responses, which resulted in the reduction of mitotic cells as well as of aberrant mitotic figures in the X-ray-irradiated *14-3-3ϵ*-null small cell lung cancer cell line. Interestingly, we also found that the G_2 checkpoint response is frequently impaired to various degrees in a large fraction of small cell lung cancer cell lines. These findings suggest the possible involvement of the perturbed G_2 checkpoint in the pathogenesis of this aggressive form of human lung cancers.

INTRODUCTION

Lung cancer currently claims more than 160,000 lives annually as the number one cause of cancer deaths in the United States (1), and it has also become the leading cause in Japan with more than 50,000 fatalities annually (2). A better understanding of the molecular pathogenesis of this disease is, thus, urgently needed for many nations to develop a breakthrough that would result in a drastic reduction in the number of victims. Molecular biological studies have provided clear evidence of multistep accumulation of multiple genetic defects in both tumor suppressor genes and dominant oncogenes (3). Although allelic losses are a hallmark of the presence of a tumor suppressor gene within the affected chromosomal region, previous cytogenetic and molecular biological analyses have resulted in the discovery of allelic losses in various chromosomal regions, which provides an important clue for the identification of the inactivated tumor suppressor genes in lung cancers. It is clear, however, that additional tumor suppressor genes need to be identified for a better understanding of this fatal disease. In this connection, we previously reported that, in addition to the *p53* gene at 17p13.1 (4), an as-yet-unidentified tumor suppressor gene(s) residing at 17p13.3 might also play a role in lung carcinogenesis, possibly in an earlier phase than does the *p53* gene (5–7). In addition to lung cancers, 17p13.3 appears to be frequently involved in various other types of cancers such as breast and ovarian cancers (8–11).

It is now well recognized that lung cancers frequently carry defects in the G_1 checkpoint (4), and emerging evidence indicates that the mitotic checkpoint may also play an important role (12, 13). In contrast, only very little is known about the potential involvement of G_2 checkpoint impairment in human cancers, and virtually no data are available regarding its relation to the pathogenesis of lung cancer (14, 15). Previous studies, mostly on yeast, have suggested that a coordinately controlled G_2 checkpoint prevents cells with damaged DNA from entering mitosis, thus playing an important role in the maintenance of chromosomal integrity (16, 17). The G_2 checkpoint response is mediated by multiple kinases and phosphatases, resulting in the direct augmentation of their activities as well as in changes in the subcellular localization of key molecules such as Cdc25C. Association of 14-3-3 with Cdc25C in response to phosphorylation by CHK1 and/or CHK2 is believed to trigger the nuclear-cytoplasmic transition (18–23). Among the seven 14-3-3 isoforms thus far identified, Cdc25C has been shown to bind mainly to 14-3-3ϵ in *Xenopus* egg extract (24). Although 14-3-3ϵ also forms a complex with Cdc25C in human cells (22), it is not clear which 14-3-3 isoform actually plays a significant role in the G_2 checkpoint response in this setting.

We report here the identification of a homozygous deletion of the *14-3-3ϵ* gene, which resides within the commonly deleted region at 17p13.3. In addition, the G_2 checkpoint response could be restored to a significant extent by the introduction of exogenous *14-3-3ϵ* into a SCLC[3] cell line carrying the homozygous deletion. Furthermore, we found for the first time that the G_2 checkpoint is frequently impaired in a significant fraction of SCLC cell lines, which suggests the possible involvement of the perturbed G_2 checkpoint in the pathogenesis of this aggressive form of human lung cancers.

MATERIALS AND METHODS

Cell Lines. Lung cancer lines with the prefix "ACC-LC" were established in our laboratory at Aichi Cancer Center. These included the ACC-LC-48, -49, -52, -76, -80, -87, -97, -170, and -172 SCLC cell lines as well as the ACC-LC-176 NSCLC cell line (25, 26). Other lung cancer cell lines used were A549 (purchased from the American Type Culture Collection, Manassas, VA), PC-10 (a generous gift from Dr. Y. Hayata, Tokyo Medical University, Tokyo, Japan), SK-LC-2 and Calu1 (generous gifts from Dr. L. J. Old, Memorial Sloan-Kettering Cancer Center, New York, NY) and NCI-H69 (a generous gift from Dr. J. D. Minna, University of Texas Southwestern Medical Center, Dallas, TX). HPL1D, a human epithelial cell line derived from normal peripheral lung, was also established in our laboratory, and BEAS2B, a human bronchial epithelial cell line, was kindly donated by Dr. C. C. Harris (National Cancer Institute, Bethesda, MD; Refs. 27, 28). HCT116 and TIG-112 were obtained from, respectively, the American Type Culture Collection and the Japanese Collection of Research Bioresources (Tokyo, Japan). Among the cell lines used in this study, ACC-LC-48, -49, -76, -80, -97, -172, PC-10, and Calu1 had mutant *p53*, whereas ACC-LC-170, -176, A549, and HCT-116 carried wild-type *p53* (29). As for the mitotic checkpoint, ACC-LC-176 and HCT-116 were found to be functionally normal, whereas PC-10 and Calu1 exhibited impaired response to nocodazole treatment (30).

Duplex PCR Amplification of *14-3-3ϵ* and *p53*. Duplex PCR amplification was performed by using genomic DNA, followed by electrophoresis on a 3% agarose gel. The following oligonucleotide primers were used for amplification: *14-3-3ϵ* exon 1S (sense), 5′-GAGTCGGAGACACTATCCG; *14-3-3ϵ*

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[2] To whom requests for reprints should be addressed, at the Division of Molecular Oncology, Aichi Cancer Center Research Institute, 1-1 Kanokoden, Chikusa-ku, Nagoya 464-8681, Japan.

[3] The abbreviations used are: SCLC, small cell lung cancer; NSCLC, non-SCLC.

intron 1AS (antisense), 5′-GCAGAGGGTCCGAGAATTC; *p53* exon 5S (sense), 5′-AGCAAGCTTGACTTTCAACTCTGTCTCCTT; and *p53* exon 5AS (antisense), 5′-AGCGGATCCACCAGCCCTGTCGTCTCTCCA. PCR amplification consisted of 35 cycles (95°C for 30 s, 60°C for 30 s, and 72°C for 30 s) after the initial denaturation step (95°C for 3 min). Other sequence-tagged site markers and genes screened for the presence of homozygous deletions at 17p13.3 were *D17S695*, *D17S926*, *ABR(CA)n*, *AKG2–1* (*OVCA1*; Ref. 10), *D17S5*, *HIC-M6* (*HIC-1*; Ref. 11), *P13–1/P13–2*, and *D17S379*.

Northern Blot Analysis. Northern blot analysis used a PCR-generated cDNA probe, which covered the entire open reading frame of the *14-3-3ε* gene. The primers used for probe generation were *14-3-3ε* exon 1S (sense, as above) and AS3 (antisense), 5′-TTTCTCTTGTTGGCTTATGTC. PCR amplification consisted of 35 cycles (95°C for 20 s, 55°C for 20 s, and 72°C for 1.5 min) after the initial denaturation step (95°C for 3 min).

Western Blot Analysis. Ten μg of total cell lysate solubilized in Laemmli's sample buffer was electrophoresed on a 12.5% SDS-polyacrylamide gel and transferred to an Immobilon-P membrane (Millipore Co., Bedford, MA). The filter was first incubated with anti-14-3-3ε polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and then with horseradish peroxidase-conjugated secondary antibody (Amersham Life Science, Buckinghamshire, United Kingdom). For visualization, an enhanced chemiluminescence system (Amersham) was used.

Analysis of G_2 Checkpoint Response. G_2 checkpoint response was examined essentially as described by Kaufmann *et al.* (31). Exponentially growing cells were irradiated with 1 Gy irradiation (Hitachi MBR-1520R, Hitachi, Tokyo, Japan), harvested at 1–4 h, and swollen by incubation in hypotonic media (100 mM KCl for ACC-LC-172, 75 mM KCl for other SCLC cells, and 50 mM KCl for residual cells) at room temperature for 20 min. After the addition of an equal volume of a fixative [1:3 (v/v) acetic acid:methanol], cells were centrifuged at 150 × *g* for 10 min, followed by aspiration of the supernatant. The cells were then resuspended in the fixative, and the fixation steps were repeated twice, after which the cells were dropped onto slide glasses, dried immediately, and stained with Giemsa. To measure the mitotic index (percentage of viable cells in mitosis), at least 2000 cells were counted for each measurement. At least two independent experiments were carried out in duplicate.

Induction of aberrant mitoses after X-ray irradiation was examined, essentially in the manner as described by Ianzini and Mackey (32). In brief, exponentially growing cells were irradiated with 6 Gy, harvested at 48 h, and processed with hypotonic media and fixative as described above. A minimum of 100 mitotic figures was examined microscopically to determine the proportion of aberrant mitoses. At least two independent experiments were carried out in duplicate.

***14-3-3ε* Expression Constructs.** The *14-3-3ε* cDNA fragment covering the entire coding region was generated by PCR amplification using the primers Forward (F)1 (sense), 5′-CGGAATTCCATGGATGATCGAGAGGATCT and Reverse (R)1 (antisense), 5′-GCTCTAGACTCACTGATTTTCGTCTTCC. PCR products were cloned into the *Eco*RI and *Xba*I sites of either the pcDNA3 or pcDNA3-*myc* expression vector, followed by sequencing of the entire inserts. pcDNA3-*myc* was prepared by inserting an annealed oligonucleotide encoding *myc*-tag sequence between the *Kpn*I and *Eco*RI site of pcDNA3 (Invitrogen Co., Carlsbad, CA). The *Eco*RI–*Xho*I fragment of *14-3-3ε* cDNA was isolated from a cDNA clone (the Integrated Molecular Analysis of Genomes and their Expression Clone ID: 564052), followed by further digestion with *Msp*A1I. The resulting *Msp*A1I cDNA fragment of *14-3-3ε* was then cloned into the *Eco*RV site of pIRESneo expression vectors.

Generation of Transient and Stable Transfectants of *14-3-3ε*. Transient transfection was performed with a cationic lipid reagent, DMRIE-C (Invitrogen Co.), according to the manufacturer's instructions. Briefly, ACC-LC-48 cells (2×10^7 cells) were cotransfected with 12 μg of pcDNA3-*myc*-*14-3-3ε* or the empty pcDNA3-*myc* along with 4 μg of pMACS4.1 (Miltenyi Biotec, Auburn, CA) for magnetic isolation of transfected cells. After a 6-h incubation, the cells were transferred to a standard medium supplemented with 5% FCS. Forty-eight h after transfection, the transfected cells were magnetically isolated with a MACSelect4 transfected cell selection kit (Miltenyi Biotec) and divided into two parts, one part for mitotic index examination and the other for immunohistochemical analysis with anti-*myc* 9E10 monoclonal antibody (Berkeley Antibody Co., Richmond, CA).

For the establishment of stable *14-3-3ε* transfectants, 3×10^5 cells of ACC-LC-48 were transfected by using DMRIE-C with 1 μg of either pcDNA3-*14-3-3ε* or pIRESneo-*14-3-3ε*. Their respective empty vectors and pBluescript II SK(−) were also used as vectors alone and as mock transfection controls, respectively. After a 6-h incubation, the cells were transferred to standard medium supplemented with 5% FCS. Forty-eight h after transfection, the cells were dissociated in 0.02% EDTA and resuspended in a semisolid medium containing 1.3% methylcellulose as well as 5% FCS and 400 μg/ml G418. Cells were incubated for 4 weeks until individual G418-resistant colonies could be isolated.

Determination of Growth Curves. ACC-LC-48 and the stable transfectants were dissociated in 0.02% EDTA and seeded onto 35-mm dishes at 7.5×10^4 cells/dish. The number of cells was counted every other day up to 8 days after seeding. Three independent experiments were performed in triplicate, all yielding similar results.

RESULTS

Identification of Homozygous Deletion of the *14-3-3ε* Gene. The identification of a homozygous deletion has provided an important clue to the isolation of a number of tumor suppressor genes including *RB*, *p16*, *FHIT*, *RASSF1*, *Smad4*, and *PTEN/MMAC1* (33–42). We screened 65 lung cancer cell lines with the aid of nine markers including *HIC-1* and *OVCA1*, all of which had been mapped within the commonly deleted region of lung cancers at 17p13.3. A single sequence-tagged site marker corresponding to the *14-3-3ε* gene was consequently found to yield no amplification products in either of two SCLC cell lines despite robust amplification of *p53* exon 5 in duplex PCR analysis (Fig. 1*A*). The two SCLC cell lines, ACC-LC-48 and ACC-LC-52, had been established from distinct metastatic sites of the same patient at different treatment periods, which suggests the occurrence of homozygous deletions before metastasis *in vivo*. Our preliminary analysis also indicated that the homozygous deletion does not affect the *PITPN* and *MYO1C* loci, which reside relatively close to the *14-3-3ε* gene, indicating that the extent of deletion is less than about

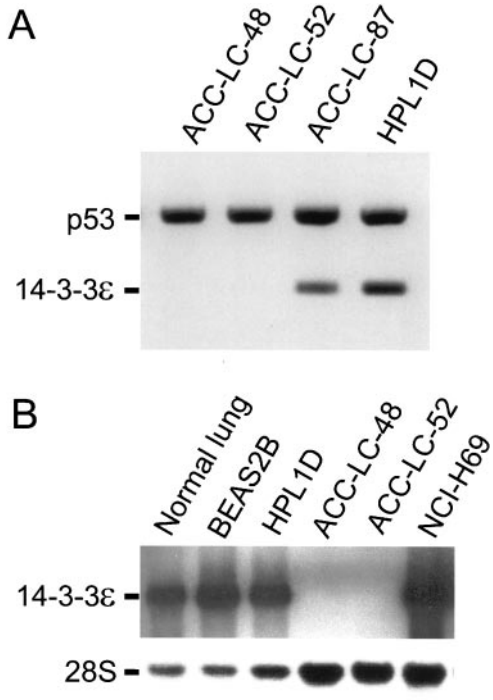

Fig. 1. Detection of homozygous deletion of the *14-3-3ε* gene in two SCLC cell lines. *A*, duplex PCR analysis showing no amplification of *14-3-3ε* despite robust amplification of *p53*. *B*, Northern blot analysis showing complete absence of *14-3-3ε* expression in the SCLC cell lines with homozygous deletion.

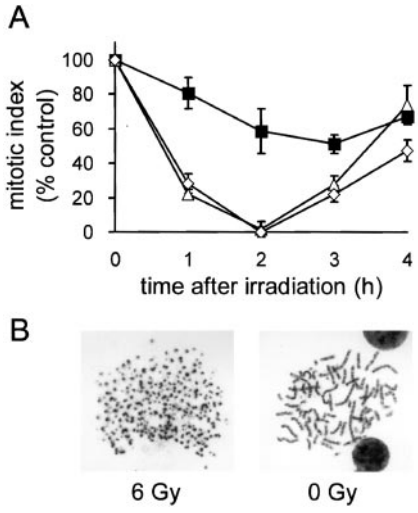

Fig. 2. Defective response to X-ray irradiation in a *14-3-3ϵ*-null SCLC cell line, ACC-LC-48. *A*, mitotic delay at various time points after treatment with 1 Gy irradiation. Mitotic fractions in irradiated samples are expressed as a percentage of mitotic fractions in nonirradiated controls. ■, ACC-LC-48; △, TIG-112; ◇, HCT116. *B*, induction of fragmented chromosomes 48 h after 6 Gy irradiation, a finding consistent with the occurrence of mitotic catastrophe.

700 kb.[4] Northern blot analysis showed complete absence of *14-3-3ϵ* expression in the two SCLC cell lines (Fig. 1*B*).

Impaired G_2 Checkpoint Response Accompanied by Induction of Aberrant Mitosis in ACC-LC-48. The identification of a homozygous deletion of *14-3-3ϵ* in the two SCLC cell lines prompted us to examine the function of the G_2 checkpoint in the *14-3-3ϵ*-null ACC-LC-48 cell line, together with that in the normal human fibroblast line TIG-112 and the colon cancer cell HCT116, which had been shown to have intact G_2 checkpoint function (43, 44). One Gy irradiation eliminated virtually all mitotic cells within a few hours in the control cell lines, whereas such a steep decline of mitotic indices was not observed in ACC-LC-48; and mitotic indices in the irradiated cells remained at >50% of nonirradiated ones. These results suggested a markedly less efficient G_2 arrest and G_2 checkpoint impairment (Fig. 2*A*).

Because it has been reported that cells with a defective G_2 checkpoint show aberrant mitosis as a consequence of untimely mitotic entry after DNA damage (43, 45), we next examined the mitotic configurations of ACC-LC-48 after irradiation. Apparently aberrant mitoses were induced resulting in the presence of fragmented chromosomes, a configuration consistent with the induction of mitotic catastrophe (Ref. 46; Fig. 2*B*). Such aberrant chromosomes were observed in 84 ± 2% of the mitotic cells with and 23 ± 2% of those without irradiation. In parallel with this observation, mitotic cells of ACC-LC-48 after irradiation, which were positive for antiphosphohistone H3 antibody staining (Upstate Biotechnology, Lake Placid, NY) were negative for the terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assay (data not shown).

Restoration of G_2 Checkpoint Response and Reduction of Aberrant Mitoses in ACC-LC-48 Resulting from Transient Transfection of *14-3-3ϵ*. We next investigated whether introduction of *14-3-3ϵ* could restore G_2 checkpoint response in ACC-LC-48. ACC-LC-48 cells were transiently transfected with a cytomegalovirus promoter-driven expression construct of *14-3-3ϵ*, followed by separation of the transfected cells with the aid of the MACSelect4 system. The resultant selected cells were then processed for both mitotic index examination for the evaluation of G_2 checkpoint response and immunohistochemical analysis for the measurement of 14-3-3ϵ positivity. Mitotic indices decreased to 31 ± 3% of the nonirradiated cells in *14-3-3ϵ* transfected cells, whereas there was no noticeable difference between empty vector-transfected cells and parental ACC-LC-48 (Fig. 3*A*).

We also investigated whether induction of aberrant mitosis could be reduced by the transient introduction of *14-3-3ϵ* into ACC-LC-48. Aberrant mitoses decreased to 57 ± 2% of all mitoses in the cells transfected with *14-3-3ϵ* 48 h after 6 Gy irradiation, and aberrant mitoses remained at 86 ± 2% of the mitoses when transfected with an empty vector (Fig. 3*B*). It was noted that 14-3-3ϵ positive cells could be separated in the transient transfection experiments only up to 52–62% purity, which suggests that the observed incomplete restoration of the G_2 checkpoint in *14-3-3ϵ* transfectants may be attributable to substantial contamination of nontransfected cells.

Reduced Growth Rate and Restoration of G_2 Checkpoint by Stable Transfection of *14-3-3ϵ*. To further confirm restoration of G_2 checkpoint response as a result of the introduction of exogenous *14-3-3ϵ* into ACC-LC-48, we isolated stably transfected clones after G418 selection in a semisolid suspension culture. The number of colonies was markedly reduced by the introduction of *14-3-3ϵ* when compared with empty vector transfection, but 18 colonies could eventually be expanded. However, none of them expressed 14-3-3ϵ at detectable levels in Western blot analyses, which suggested that constitutive overexpression of 14-3-3ϵ may be incompatible with SCLC cell growth (data not shown).

We next used the pIRESneo expression vector instead of pcDNA3, so that *14-3-3ϵ* expression could be placed under regulation of the same promoter that also drives the neomycin resistance gene. Again, very few colonies expanded after selection with G418, but eventually three 14-3-3ϵ-positive clones could be obtained. However, the 14-3-3ϵ expression levels of all three of the clones were significantly lower than those of endogenous 14-3-3ϵ in other SCLC cell lines such as ACC-LC-170 (Fig. 4*A*). Comparison of the growth rate of the three stable, *14-3-3ϵ*-expressing clones with empty pIRESneo vector control clones, as well as with the parental ACC-LC-48, revealed significantly slower growth of the *14-3-3ϵ* transfectants (Fig. 4*B*). This

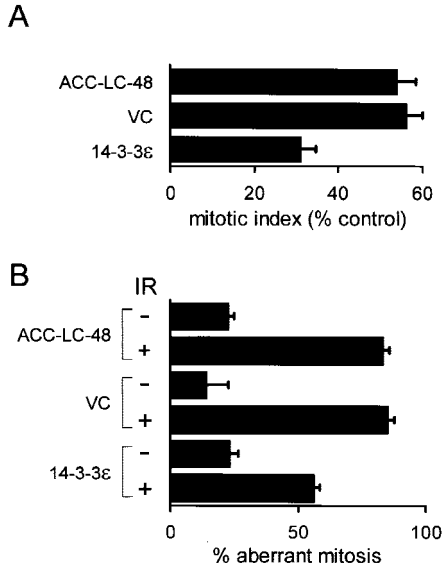

Fig. 3. Restoration of G_2 checkpoint responses in transient *14-3-3ϵ* transfectants of ACC-LC-48. *A*, mitotic fractions of transient transfectants 3 h after exposure to 1 Gy irradiation. *B*, frequency of occurrence of aberrant mitoses after 6 Gy irradiation in transient transfectants.

[4] Unpublished observations.

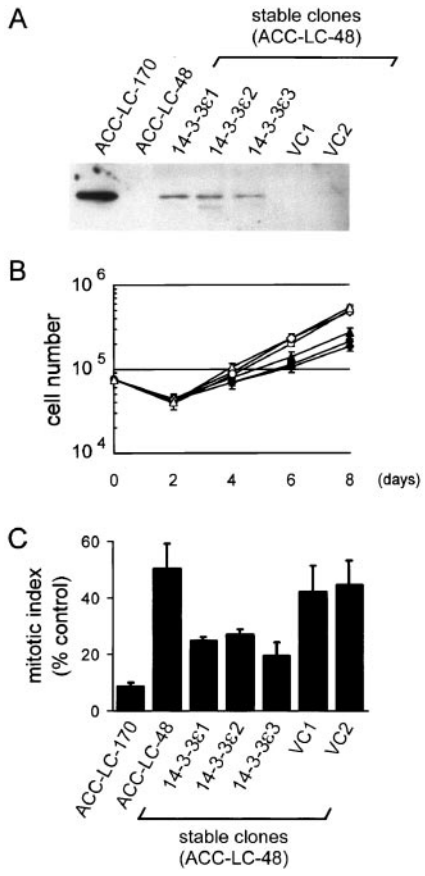

Fig. 4. Restoration of G_2 checkpoint responses in stable *14-3-3ϵ* transfectants of ACC-LC-48. *A*, Western blot analysis with anti-14-3-3ϵ antibody showing low-level expression in three stable transfectants. *B*, growth curves of the *14-3-3ϵ* transfectants (14-3-3ϵ1 to -3) in comparison with empty vector transfectants (VC1 and -2) and parental ACC-LC-48. ●, 14-3-3ϵ1; ◆, 14-3-3ϵ2; ▲, 14-3-3ϵ3; ○, VC1; ◇, VC2; △, ACC-LC-48. *C*, mitotic fractions of stable transfectants 3 h after irradiation with 1 Gy of X-ray.

suggests that 14-3-3ϵ may function as a negative growth regulator in SCLC cells. With regard to G_2 checkpoint response, mitotic indices were measured with or without 1 Gy irradiation in *14-3-3ϵ* and vector control clones as well as in the parental ACC-LC-48. All of the three *14-3-3ϵ* transfectants exhibited incomplete but significant restoration of the G_2 checkpoint response, in contrast to the absence of any significant differences between vector control clones and ACC-LC-48 (Fig. 4*C*). Combined with the findings obtained with the transient transfection experiment, these observations indicated that the homozygous loss of *14-3-3ϵ* perturbed the G_2 checkpoint response to X-irradiation in ACC-LC-48.

Frequent Impairment of G_2 Checkpoint Response in SCLC Cell Lines. We next investigated whether impairment of the G_2 checkpoint response might be a common feature of SCLCs. Mitotic indices were examined after exposure to 1 Gy irradiation in an additional seven SCLC cell lines as well as in ACC-LC-48, and also in four NSCLC cell lines and two control cell lines (*i.e.*, TIG-112 and HCT116). Interestingly, the majority of SCLC cell lines showed a considerably perturbed response. In fact, in contrast to the virtually complete disappearance of mitotic cells in all of the four NSCLC and two control cell lines, the mitotic indices of irradiated cells of four of the eight SCLC cell lines remained higher than 25% of those of the corresponding nonirradiated cells (Fig. 5). These results suggested significant perturbation of the G_2 checkpoint response in a large fraction of SCLC cell lines.

DISCUSSION

In the study presented here, we found that the *14-3-3ϵ* gene, which resides within the commonly deleted region at 17p13.3 in lung cancers, was homozygously deleted in two SCLC cell lines originating from the same patient at different treatment periods. ACC-LC-48 with the homozygous deletion showed an abnormal G_2 checkpoint response to ionizing radiation including frequent induction of aberrant mitosis, whereas the introduction of *14-3-3ϵ* significantly, although incompletely, restored the impairment. This indicated that 14-3-3ϵ plays a part in the G_2 checkpoint in human cancer cells. The incomplete restoration of the G_2 checkpoint response may be attributable to its observed inability to achieve sufficient 14-3-3ϵ expression. Alternatively, it is possible that an additional molecule(s) that is important for the G_2 checkpoint function may have been altered in this cell line. In this regard, we noted that although it has been suggested that p53 plays a role in sustaining G_2 arrest in response to DNA damage (47), the presence of *p53* mutations was not necessarily associated with impaired induction of G_2 arrest as an early response to irradiation.

The G_2 checkpoint is one of the most highly conserved mechanisms that regulate the cell cycle by preventing damaged cells from causing improper progression of cell cycle (16, 17). Detailed studies on yeast have provided considerable evidence that a coordinately controlled G_2

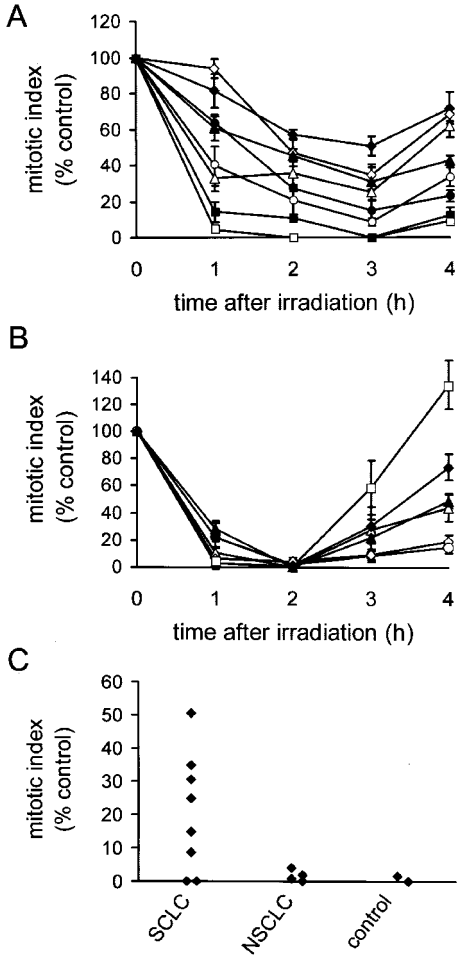

Fig. 5. Significant fraction of cells entering mitosis after 1 Gy of irradiation in SCLC cell lines (*A*) in contrast to nearly complete elimination of mitotic cells in NSCLC and control cell lines (*B*). In *A*: ◆, ACC-LC-48; ◇, ACC-LC-80; ▲, ACC-LC-49; △, ACC-LC-76; ●, SK-LC-2; ○, ACC-LC-170; ■, ACC-LC-172; and □, ACC-LC-97. In *B*: □, A549; ◇, ACC-LC-176; ○, PC-10; △, Calu1; ◆, TIG-112; and ▲, HCT116. *C*, comparison of lowest mitotic indices over time, showing significantly reduced response to irradiation in SCLC cell lines.

checkpoint is important for the maintenance of chromosomal integrity. A possible link between inactivation of the G_2 checkpoint function and acquisition of chromosomal abnormality has also been demonstrated in mammalian cells (31, 48). Human fibroblasts derived from ataxia-telangiectasia cases carry a defective G_2 checkpoint in conjunction with increased radiosensitivity and frequent chromosomal aberrations (49). Whereas 14-3-3σ, another member of the 14-3-3 family, plays a role in G_2 checkpoint response to DNA damage by excluding the cdc2-cyclin B1 mitotic initiation complex from nucleus, a mechanism distinct from that of 14-3-3ϵ, somatic knockout cells of *14-3-3*σ show loss of the normal G_2 checkpoint response to DNA damage and the accumulation of chromosomal aberrations (43, 44). Lung cancers are well known to carry complex chromosomal abnormalities including multiple numerical and structural alterations, and we recently obtained direct evidence of the pervasive presence of the chromosomal instability phenotype in lung cancer cell lines (30). Our finding that a significant fraction of SCLCs, which are very sensitive to irradiation and chemotherapy and at the same time the most aggressive type of lung cancers, exhibited an abnormal G_2 checkpoint response is, thus, of great interest not only from a biological point of view but also in terms of clinical implications. Our preliminary examination of the *14-3-3*ϵ gene in the panel of SCLC cell lines used for this study did not disclose any additional genetic alterations or changes in its transcription level (data not shown). Some possibility remains of the occurrence of abnormalities in SCLC, such as perturbation of the amount of the 14-3-3ϵ protein that is attributable to increased degradation and functional inactivation (caused by altered modification) that leads to aberrations in protein interaction. Epigenetic inactivation of *14-3-3*σ caused by aberrant hypermethylation was recently reported in breast cancer, gastric cancer, and hepatocellular carcinomas (14, 15, 50), and we recently found that CHK2, a key kinase involved in the G_2 checkpoint pathway, was somatically mutated in a small fraction of SCLCs (51, 52). Therefore, the search for additional molecule(s) that may account for the high frequency of G_2 checkpoint aberrations in SCLC cell lines, also appears to be warranted.

Lastly, the possibility remains that an as-yet-unidentified gene(s) may also be involved in the homozygous deletion and that the resultant inactivation may have other biological consequences in terms of lung carcinogenesis. In this regard, the presence of multiple cancer-related genes in close vicinity has precedents in *MLH1* and *RASSF1A* at 3p21.3, *Smad2* and *Smad4* at 18q21.1, and *MCC* and *APC* at 5q21. Additional detailed characterization of the homozygous deletion reported here may, therefore, lead to a better understanding of the molecular pathogenesis of lung cancer.

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Hiroyuki Konishi, Taku Nakagawa, Tomoko Harano, et al.

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