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Increased Radiosensitivity of p16 Gene-deleted Human Glioma Cells after Transfection with Wild-type p16 Gene

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The A1235 and T98 cell lines derived from human gliomas have homozygous deletions in their p16 genes and are radiosensitive and radioresistant, respectively, with respect to other established glioma cell lines. These differences in radiosensitivity may be due to variations to some extent among cell lines, rather than genetically defined resistance or sensitivity. We examined the effect on radiation sensitivity of introducing a wild-type p16 gene into both p16-deficient glioma cell lines. The plasmid pOPMTS containing human wild-type p16 cDNA and a neomycin resistance gene, or the control plasmid pOPRSV1, were transfected into these cells. Clones from both cell lines, which expressed wild-type p16 mRNA constitutively after transfection with pOPMTS, were more radiosensitive than the parental cells and clones obtained after transfection with the negative control plasmid.

Key words: Radiosensitivity — Wild-type p16 — Human glioma cell line

The p16 gene ($p16^{INK4a}$ or MTS1) is located on chromosome 9p21,[1] a region showing frequent loss of heterozygosity in malignant gliomas,[2,3] and encoding the p16 protein, an inhibitor of cyclin D/cyclin-dependent kinase 4 (CDK4).[4] The main function of the cyclin D/CDK4 complexes may be to phosphorylate the retinoblastoma (Rb) protein in late G_1.[5,6] Inactivation of p16 function has been linked to the development of many types of neoplasms, and recent investigations have provided insight regarding the manner in which the inactivation may contribute to cell proliferation.[7] The p16 gene is often mutated or homozygously deleted in certain types of tumors and tumor-derived cell lines.[8–10] These results have been interpreted to imply that p16 is a tumor suppressor gene.

Human malignant gliomas are radioresistant tumors.[11] Frequent p16 mutations (mainly deletions)[8,12] and loss of p16 expression[13] occur in human malignant glioma cell lines. The A1235 and T98 cell lines derived from human malignant gliomas have homozygous deletions in their p16 genes[12] and are radiosensitive and radioresistant, respectively, with respect to other established glioma cell lines.[14] These differences in radiosensitivity may be due to variations to some extent among cell lines, rather than genetically defined resistance or sensitivity, or it may be that there are multiple genetic differences between T98 and A1235 cell lines that influence radiosensitivity. To assess the role of p16 in cellular response to ionizing radiation, we examined the effect on radiation sensitivity of introducing a wild-type p16 gene into p16-deficient glioma cells.

MATERIALS AND METHODS

Cell lines and culture conditions The established human glioma cell lines, A1235 and T98, which have a deletion in the coding region of the p16 gene,[12] were used. The cells are deficient in p16 expression and lack the functional p16 protein. For the positive control, the MOO6 cell line that has an intact p16 gene was used.[12] The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum (FBS) (Hyclone Laboratories, Inc., Logan, UT) at 37°C with 95% air and 5% CO_2.

Plasmids and transfection p16 cDNA was amplified from the total RNA of normal human skin fibroblasts by a reverse transcriptase-polymerase chain reaction (RT-PCR) using the Super Script Preamplification System (Life Technologies, Gaithersburg, MD) with the primers 5′-AATTCGGCACGAGGCAGCATGGAGCCTTCG-3′ and 5′-GCCCTGTAGGACCTTCGGTGACTGAT-GATC-3′. An expression plasmid for p16, pOPMTS, was constructed as follows. A plasmid pOPRSV1 containing Rous sarcoma virus long terminal repeats and neomycin resistance (neo^r) gene was digested with the Not I restriction enzyme. After we had confirmed by DNA sequencing that the PCR product did correspond to a wild-type p16 cDNA, the cDNA was ligated to Not I linkers. Then,

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the human normal p16 cDNA was ligated into the Not I site of pOPRSV1. The plasmid pOPRSV1, without p16 cDNA, was used as a control.

Approximately 10^6 cells were transfected with 5 μg of pOPMTS or pOPRSV1 by electroporation with the unit (model X-CELL 2000, pds Inc., Madison, WI) operated at 800 V. The cells were cultured in DMEM containing 10% FBS for 36 to 48 h, and then in medium containing G418 (400–800 μg/ml). The cultures were maintained for 2 to 3 weeks until colonies formed. The transfection frequencies for both pOPMTS and pOPRSV1 were 2 to 8×10^{-4}.

Cell growth, survival and x-irradiation The growth rate of cells in log phase was estimated from growth curves. The cell number per plate was measured using a Coulter counter (model ZM, Coulter Electronics Ltd., Luton, Beds, England). Methods of x-irradiation and the analysis of cell survival were as described previously.[15, 16] Five replicate plates per experiment were used for each survival point and the experiments were repeated at least three times. Cell survival was statistically analyzed by the two-way analysis of variance and by the *t* test for unpaired data.

Northern analysis Details of northern analysis have been described elsewhere.[16, 17] The p16 cDNA obtained by RT-PCR was used as a probe. The β-actin probe was a 0.5 kb *Hin*fI fragment including exons 3 and 4 of the human *β-actin* gene.[18] The densitometric analysis was done using NIH Imaging 1.54.

Flow cytometry Cells were suspended in phosphate-buffered saline (PBS) containing 0.1% Triton X-100 after having been washed twice with PBS. RNase (1 mg/ml) (Sigma, St. Louis, MO) was added to the cell suspension and the cells were incubated for 30 min at 37°C. Propidium iodide (Molecular Probes, Inc., Eugene, OR) was added at a final concentration of 50 μg/ml and the cell suspension was left at 4°C for 1 h. The cells were filtered through 50 μm nylon mesh before flow cytometry with a FACScan system (Becton Dickinson, Co., Ltd., Franklin Lakes, NJ).

Western immunoblots Details of western immunoblotting have been described elsewhere.[14] A polyclonal anti-p16 antibody (15126E, PharMingen, San Diego, CA) and a monoclonal anti-pRb antibody (IF8, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used. Autoradiography with enhanced chemiluminescence was done according to the instructions supplied by the manufacturer (Amersham International plc, Buckinghamshire, UK).

RESULTS

Isolation of clones expressing p16 mRNA Forty and 20 clones of A1235 and T98, respectively, were isolated from the transfected cell population after selection with G418. Eleven clones from A1235 and 6 clones from T98 cells expressed p16 mRNA. Among these clones, we randomly selected 8 clones (4 from each line) for experiments. The growth rates of these clones were not markedly different from those of the parental and negative control cells (data not shown). Fig. 1 shows the p16 northern analysis of the parental cells (A1235 and T98), the negative controls (A-V3 and T-V4), and the clones transfected with p16. Clones derived from A1235 cells (A-C11, A-C20, A-C29 and A-C31) and T98 cells (T-C2, T-C5, T-C12 and T-C15) expressed p16 mRNA constitutively. From the western blot analysis, however, p16 protein could not be clearly detected in these clones compared with that in the positive control MOO6 lines (Fig.2 and data not shown).

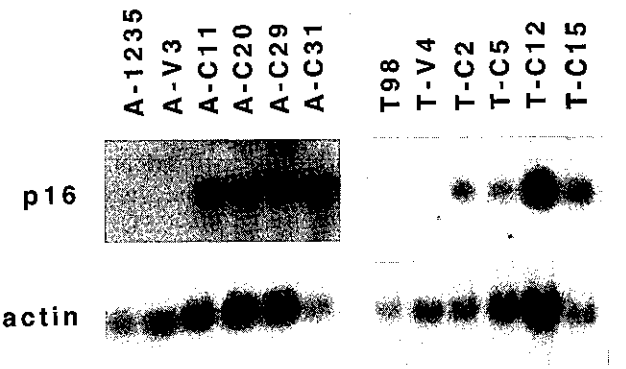

Fig. 1. Northern blot analysis of p16 and β-actin in the parental A1235 and T98 lines, the negative control A-V3 and T-V4 lines, and clones (A-C11, A-C20, A-C29, A-C31, T-C2, T-C5, T-C12 and T-C15) obtained after transfection with the plasmid pOPMTS. For the negative controls, the plasmid pOPRSV1, which did not harbor p16 cDNA, was used for transfection. Total RNA was electrophoresed, transferred and hybridized with probes for p16 or β-actin sequences.

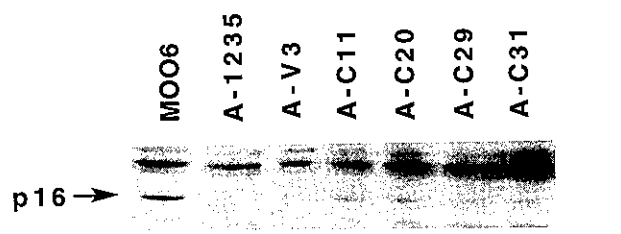

Fig. 2. Western blot analysis of p16 in the parental A1235 line, the negative control A-V3, clones expressing p16 mRNA (A-C11, A-C20, A-C29 and A-C31), and the positive control MOO6 line.

Radiation sensitivity of clones expressing p16 mRNA The radiation sensitivity of clones expressing p16 mRNA was examined. Fig. 2 shows x-ray survival curves of the parental cell lines (A1235 and T98), the negative controls produced by transfection with pOPRSV1 (A-V3 and T-V4), the clones lacking p16 mRNA expression (A-C10 and T-C8), and the clones expressing p16 mRNA (A-C11, A-C20, A-C29, A-C31, T-C2, T-C5, T-C12 and T-C15). A1235 cells were relatively radiosensitive and T98 cells were radioresistant. The *Do* values for A1235 and T98 cells were 1.05 and 2.03 Gy, respectively. In both cell lines, all clones expressing p16 mRNA were significantly radiosensitive in comparison with the parental cells and the negative controls ($P<0.01$ for A1235, A-V3 and A-C10 vs. A-C11, A-C20, A-C29 and A-C31; $P<0.05$ for T98, T-V4 and T-C8 vs. T-C2, T-C5, T-C12 and T-C15). To compare the magnitude of p16 expression with the extent of sensitization to x-rays, the ratio of the p16 band intensity to that for β-actin for each sample was estimated from Fig. 1. The ratio was 1.0 (A-C20), 1.22 (A-C11), 1.26 (A-C29) and 9.60 (A-C31) for clones derived from A1235 and 1.0 (T-C5), 1.68 (T-C2), 2.64 (T-C12) and 4.89 (T-C15) for clones derived from T98. However, the magnitude of p16 expression was not proportional to the extent of increased radiosensitivity.

Cell cycle distribution after x-irradiation The distribution of cells within the cell cycle was examined in A1235, A-V3 and A-C29 clones after x-irradiation (3 Gy). Exponentially growing cells were exposed to 3 Gy of x-rays and harvested at 20 h and 40 h after exposure. Fig. 3 shows histograms of the DNA content. A decrease in the S phase fraction and an increase in the G_2+M fraction following x-irradiation were observed in all of the cell lines. An arrest in G_2 clearly appeared and the G_1 fraction did not change markedly, indicating that G_1 arrest also occurred in these cell lines. The DNA histogram of A-C29 cells at 40 h after x-irradiation shows that a portion of the DNA was degraded (Fig. 3). Other p16-expressing clones derived from A1235 cells showed a trend similar to that of A-C29 cells (data not shown).

DISCUSSION

Eight clones that expressed the exogenously introduced wild-type p16 became sensitive to x-rays compared with their parental cell lines and the negative control clones lacking the p16 gene homozygously (Figs. 1 and 3). Recently, we observed that human melanoma cell lines lacking the p16 gene homozygously or lacking p16 mRNA expression were resistant to ionizing radiation, while those having wild-type p16 showed high sensitivity to radiation (Matsumura *et al.* unpublished data). Our findings in this study are consistent with the observations on melanoma cell lines, suggesting that p16 may be one of the factors controlling cellular radiosensitivity. From the western blot analysis, however, p16 protein could not be clearly detected in these clones compared with that in

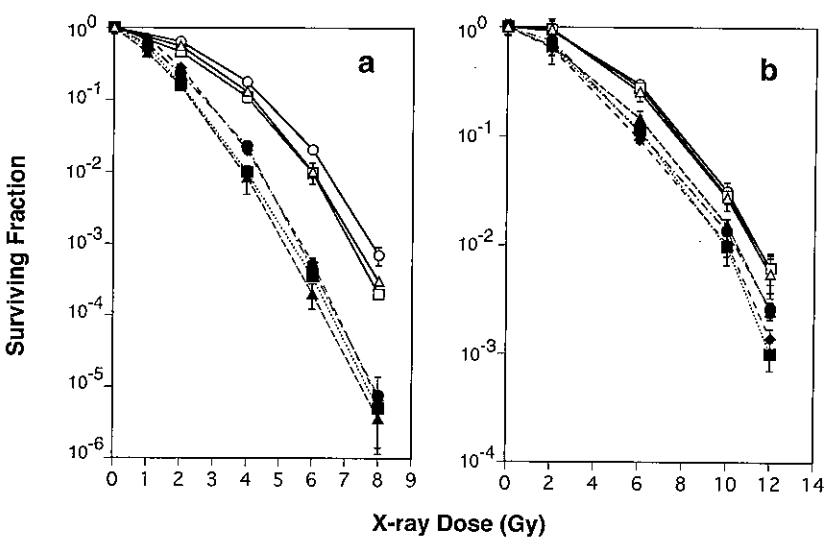

Fig. 3. X-ray survival curves of the parental cell lines (A1235 and T98), the negative controls prepared with the pOPRSV1 vector (A-V3 and T-V4), the clones lacking p16 mRNA expression (A-C10 and T-C8), and the clones expressing p16 mRNA (A-C11, A-C20, A-C29, A-C31, T-C2, T-C5, T-C12 and T-C15) obtained after transfection with the plasmid pOPMTS. Error bars represent standard deviation. a, ○ A1235, □ A-V3, △ A-C10, ▲ A-C11, ◆ A-C20, ● A-C29 and ■ A-C31. b, ○ T98, □ T-V4, △ T-C8, ▲ T-C2, ◆ T-C5, ● T-C12 and ■ T-C15.

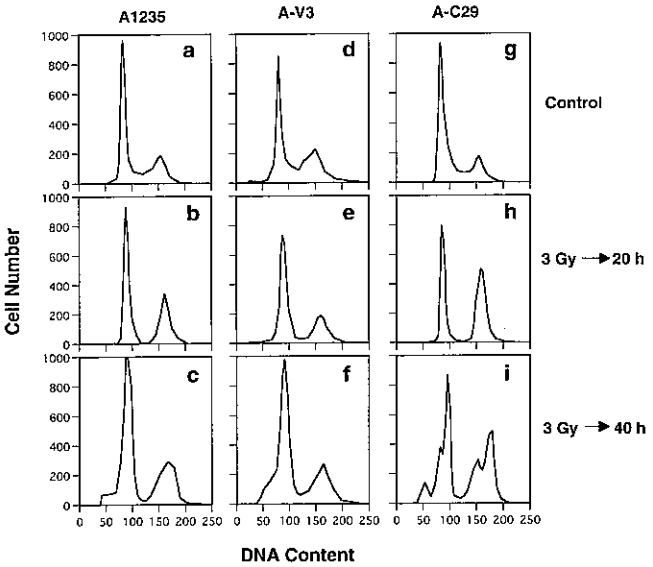

Fig. 4. Cell cycle analysis by quantitative flow cytometry of A1235, A-V3 and A-C29 cells. (a)–(c), A1235 cells; (d)–(f), A-V3 cells; (g)–(i), A-C29 cells; (a), (d) and (g), no treatment; (b), (e) and (h), 3 Gy x-ray irradiation followed by 20 h incubation; (c), (f) and (i), 3 Gy x-rays followed by 40 h incubation. Each experiment was repeated three times. The mean value (%) of cells in each peak (G_1, S and G_2+M) is as follows: (a) 47%, 40%, 13%; (b) 46%, 20%, 34%; (c) 55%, 15%, 30%; (d) 40%, 41%, 19%; (e) 46%, 25%, 29%; (f) 50%, 19%, 31%; (g) 44%, 38%, 18%; (h) 43%, 11%, 46%; (i) 40%, 18% and 42%.

the positive control MOO6 lines (Fig. 2). Turnover of the exogenous p16 proteins may be rapid in these clones.

p16 is known to be a component of the cell cycle regulation system, because overexpression of p16 arrests cell cycle progression by inhibiting the catalytic activity of the cyclin D/CDK4 complex, an inhibition that results in blocked Rb phosphorylation.[19, 20] Arap *et al.*[21] showed that transfection by wild-type p16 suppressed the growth of p16-deleted glioma cell lines (T98G, U-87MG, and U-251MG) while no effect of the p16 transfection on cell growth was observed in glioma cells having wild-type p16 (LN-319 and LN-Z308). Their results in T98G, a subline of T98, are inconsistent with ours. A possible explanation is that their method is very different from ours, in that their results were obtained with the bulk population of transfected cells. Since there is clonal diversity among transfections in studies investigating determinants of cellular radiosensitivity,[22] we analyzed multiple clones independently to determine radiosensitivity. In our study, the cell cycle phase distribution and the growth rate of clones expressing wild-type p16 mRNA showed little difference from those in the parental and the negative control cells (Fig. 4 and data not shown). Furthermore, from the western blot analysis, the phosphorylation of the Rb protein in p16-expressing clones (A-C29 and T-C12) did not change markedly in comparison with that in the parental cells or the negative control (data not shown). From our previous observations, T98 cells have a mutation in the p53 gene, a G-to-A transition in the third position of codon 237,[18] and A1235 cells have no interferon α gene and a rearrangement in the interferon β gene located near the p16 gene.[23] Therefore, even if wild-type p16 cDNA were introduced, the cell cycle regulation of these clones might not be restored.

Cellular radiation sensitivity is dependent on cell cycle phase.[24, 25] From the cell cycle analysis, the proportion of cells in the radioresistant S phase, as well as the relatively radiosensitive G_2+M phases, in A-C29 cells in the cell cycle did not change markedly as compared with that in A1235 and A-V3 cells (Fig. 4, a, d and g). The cell cycle phase distribution of T-C15 and T98 cells also showed similar trends (data not shown). The cell cycle distribution cannot account for the enhanced sensitivity to ionizing radiation observed in A-C29 and T-C15 cells. From the histograms of DNA content, a slight degradation of DNA was observed at 40 h after x-irradiation in A-C29 cells (Fig. 4 i). The increased sensitivity to x-rays might be partly due to such degradation. The degraded DNA was not observed in T-C15 cells (data not shown). These observations might be associated with the difference in the extent of x-ray sensitization between A1235 and T98 (Fig. 3).

In the treatment of cancer, sensitivity or resistance of tumor cells to ionizing radiation has substantial clinical consequences. However, the molecular mechanisms and/or intrinsic factors controlling cellular radiosensitivity are not well understood in mammalian cells. In the present study, introduction of wild-type p16 increased the radiosensitivity of both radiosensitive (A1235) and radioresistant (T98) glioma cell lines with homozygous deletions of the p16 gene. This finding suggests that exogenous wild-type p16 might be one of the factors determining cellular radiosensitivity. Restored p16 expression was associated with increased radiosensitivity in at least two glioma lines with p16 deletions. However, an association with deleted p16 or an association with tumor type remains a possibility. The exact role(s) of p16 in determining cellular sensitivity to ionizing radiation should be further investigated.

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REFERENCES

1) Okamoto, A., Demetrick, D. J., Spillare, E. A., Hagiwara, K., Hussain, S. P., Bennett, W. P., Forrester, K., Gerwin, B., Serrano, M., Beach, D. H. and Harris, C. C. Mutations and altered expression of $p16^{INK4}$ in human cancer. *Proc. Natl. Acad. Sci. USA,* **91,** 11045–11049 (1994).
2) Olopade, O. I., Jenkins, R. B., Ransom, D. T., Malik, K., Pomykala, H., Nobori, T., Cowan, J. M., Rowley, J. D. and Dias, M. O. Molecular analysis of deletions of the short arm of chromosome 9 in human gliomas. *Cancer Res.,* **52,** 2523–2529 (1992).
3) James, C. D., He, J., Carlbom, E., Nordenskjold, M., Cavenee, W. K. and Collins, V. P. Chromosome 9 deletion mapping reveals interferon α and interferon β-1 gene deletions in human glial tumors. *Cancer Res.,* **51,** 1684–1688 (1991).
4) Serrano, M., Hannon, G. J. and Beach, D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature,* **366,** 704–707 (1993).
5) Matsushime, H., Quelle, D. E., Shurtleff, S. A., Shibuya, M., Sherr, C. J. and Kato, J. D-Type cyclin-dependent kinase activity in mammalian cells. *Mol. Cell. Biol.,* **14,** 2066–2076 (1994).
6) Meyerson, M. and Harlow, E. Identification of G1 kinase activity for cdk6, a novel cyclin D partner. *Mol. Cell. Biol.,* **14,** 2077–2086 (1994).
7) Jin, X., Nguyen, D., Zhang, W.-W., Kyritsis, A. P. and Roth, J. A. Cell cycle arrest and inhibition of tumor cell proliferation by the $p16^{INK4}$ gene mediated by an adenovirus vector. *Cancer Res.,* **55,** 3250–3253 (1995).
8) Kamb, A., Gruis, N. A., Weaver-Feldhaus, J., Liu, Q., Harshman, K., Tavtigian, S. V., Stockert, E., Day, R. S., III, Johnson, B. E. and Skolnick, M. H. A cell cycle regulator potentially involved in genesis of many tumor types. *Science,* **264,** 436–440 (1994).
9) Nobori, T., Miura, K., Wu, D. J., Lois, A., Takabayashi, K. and Carson, D. A. Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. *Nature,* **368,** 753–756 (1994).
10) Mori, T., Miura, K., Aoki, T., Nishihira, T., Mori, S. and Nakamura, Y. Frequent somatic mutation of the MTS1/CDK4I (multiple tumor suppressor/cyclin-dependent kinase 4 inhibitor) gene in esophageal squamous cell carcinoma. *Cancer Res.,* **54,** 3396–3397 (1994).
11) Karlsson, U. L., Leibel, S. A., Wallner, K., Davis, L. W. and Brady, L. W. "Principles and Practice of Radiation Oncology," 2nd Ed., ed. C. A. Perez and L. W. Brady, pp. 515–552 (1992). J. B. Lippincott Co., Philadelphia.
12) He, J., Allen, J. R., Collin, V. P., Allalunis-Turner, J., Godbout, R., Day, R.S., III. and James, C. D. CDK4 amplification is an alternative mechanism to p16 gene homozygous deletion in glioma cell lines. *Cancer Res.,* **54,** 5804–5807 (1994).
13) Nishikawa, R., Furnari, F. B., Lin, H., Arap, W., Berger, M. S., Cavennee, W. K. and Huang, H.-J. S. Loss of p16-INK4 expression is frequent in high grade gliomas. *Cancer Res.,* **55,** 1941–1945 (1995).
14) Yamagishi, N., Miyakoshi, J., Ohtsu, S., Day, R. S., III. and Takebe, H. Modification of the radiosensitivity of human cells to which simian virus 40 T-antigen was transfected. *J. Radiat. Res.,* **36,** 239–247 (1995).
15) Miyakoshi, J., Ohtsu, S., Tatsumi-Miyajima, J. and Takebe, H. A newly designed experimental system for exposure of mammalian cells to extremely low frequency magnetic fields. *J. Radiat. Res.,* **35,** 26–34 (1994).
16) Shung, B., Miyakoshi, J. and Takebe, H. X-ray-induced transcriptional activation of c-myc and XRCC1 genes in ataxia telangiectasia cells. *Mutat. Res.,* **307,** 43–51 (1994).
17) Miyakoshi, J., Scudiero, D. A., Allalunis-Turner, J. and Day, R. S., III. The sensitivities of SV40-transformed human fibroblasts to monofunctional and DNA-crosslinking alkylation agents. *Mutat. Res.,* **254,** 55–64 (1991).
18) Nakajima-Iijima, S., Hamada, H., Reddy, P. and Kakunaga, T. Molecular structure of the human cytoplasmic β-actin gene: interspecies homology of sequences in the introns. *Proc. Natl. Acad. Sci. USA,* **82,** 6133–6137 (1985).
19) Guan, K.-L., Jenkins, C. W., Li, Y., Nichols, M. A., Wu, X., O'Keefe, C. L., Matera, A. G. and Xiong, Y. Growth suppression by p18, a $p16^{INK4/MTS1}$- and $p14^{INK4B/MTS2}$-related CDK6 inhibitor, correlates with wild-type pRb function. *Genes Dev.,* **8,** 2939–2952 (1994).
20) Lukas, J., Parry, D., Aagaard, L., Mann, D. J., Bartkova, J., Strauss, M., Peters, G. and Bartek, J. Retinoblastoma-protein-dependent cell cycle inhibition by the tumour suppressor p16. *Nature,* **375,** 503–506 (1995).
21) Arap, W., Nishikawa, R., Furnari, F. B., Cavenee, W. K. and Huang, H.-J. S. Replacement of the p16/CDKN2 gene suppresses human glioma cell growth. *Cancer Res.,* **55,** 1351–1354 (1995).
22) Pardo, F. S., Bristow, R. G., Taghian, A., Ong, A. and Borek, C. Role of transfection and clonal selection in mediating radioresistance. *Proc. Natl. Acad. Sci. USA,* **88,** 10652–10656 (1991).
23) Miyakoshi, J., Dobler, K. D., Allalunis-Turner, J., McKean, J. D. S., Petruk, K., Allen, P. B. R., Aronyk, K. N., Weir, B., Huyser-Wierenga, D., Fulton, D., Urtasun, R. C. and Day, R. S. III. Absence of IFNA and IFNB genes from human malignant glioma cell lines and lack of correlation with cellular sensitivity to interferon. *Cancer Res.,* **50,** 278–283 (1990).
24) Terasima, R. and Tolmach, L. J. X-ray sensitivity and DNA synthesis in synchronous populations of HeLa cells. *Science,* **140,** 490–492 (1963).
25) Sinclair, W. K. and Morton, R. A. X-ray sensitivity during the cell generation cycle of cultured Chinese hamster cells. *Radiat. Res.,* **29,** 450–474 (1966).