γ-Irradiation Deregulates Cell Cycle Control and Apoptosis in Nevoid Basal Cell Carcinoma Syndrome-derived Cells

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The nevoid basal cell carcinoma syndrome (NBCCS) is an autosomal dominant disorder characterized by nevi, palmar and plantar pits, falx calcification, vertebrate anomalies and basal cell carcinomas. It is well known in NBCCS that γ-irradiation to the skin induces basal cell carcinomas or causes an enlargement of the tumor size, although the details of the mechanism remain unknown. We have established lymphoblastoid cell lines from three NBCCS patients, and we present here the first evidence of abnormal cell cycle and apoptosis regulations. A novel mutation (single nucleotide deletion) in the coding region of the human patched gene, PTCH, was identified in two sibling patients, but no apparent abnormalities were detected in the gene of the remaining patient. Nevertheless, the three established cell lines showed similar features in the following analyses. Flow cytometric analyses revealed that the NBCCS-derived cells were accumulated in the G2M phase after γ-irradiation, whereas normal cells showed cell cycle arrest both in the G0G1 and G2M phases. The fraction of apoptotic cells after γ-irradiation was smaller in the NBCCS cells. The level of p27 expression markedly decreased after γ-irradiation in the NBCCS cells, although the effects of the irradiation on the expression profiles for p53, p21 and Rb did not differ in normal and NBCCS cells. These findings may provide a clue to the molecular mechanisms of tumorigenesis in NBCCS.

Key words: NBCCS — Cell cycle — γ-Irradiation — p27

The nevoid basal cell carcinoma syndrome (NBCCS), also known as the Gorlin syndrome or basal cell nevus syndrome, is an autosomal dominant disorder that predisposes to both developmental defects and cancer.1) NBCCS patients have nevi, palmar and plantar pits, vertebral anomalies and basal cell carcinomas. It contains 23 exons spanning approximately 35 kb and is predicted to encode a 1450 amino-acid protein containing 12 transmembrane-spanning domains and 2 large extracellular loops. Most mutations identified to date in NBCCS patients and in related tumors are a small deletion or insertion in the coding region which results in the formation of a premature protein.1–12) This suggests that a reduction in the PTCH gene leads to the developmental abnormalities observed in the syndrome and that complete loss of the patched function contributes to tumor formation.13)

The Drosophila ptc gene has been shown to function in the hedgehog signal pathway to form the anterioposterior axis of embryonic segments and larval imaginal discs, and the human PTCH gene has been revealed to have a similar function. The PTCH product is a receptor for Sonic hedgehog (SHH), a secreted molecule implicated in the formation of embryonic structures as well as tumorigenesis, and interacts with another transmembrane protein, smoothened (SMO).9, 13) According to the proposed model, the PTCH and SMO proteins form a stable complex in the cell membrane in the absence of the signal. When the Sonic hedgehog signal acts, SHH binds to PTCH, the complex is dissociated and free SMO plays a role in transducing the signal in the successive step.
Although NBCCS patients are sensitive to induction of tumor developments by γ-irradiation, studies on the survival of NBCCS-derived cells after irradiation have yielded conflicting results, with the cells being more sensitive than, or no different from normal cells. We previously reported a transient acceleration of DNA synthesis with no reduction in the cell survival after γ-irradiation of fibroblasts obtained from NBCCS patients, suggesting the existence of an unusual cellular response, rather than simple killing. Heterozygous ptc gene knockout mice not only developed several features observed in the NBCCS patients, such as generalized overgrowth and a variety of neural and skeletal anomalies, but also had a high incidence of embryonal rhabdomyosarcomas. The mice also exhibited an increased sensitivity to γ-radiation, resulting in the development of anomalies and tumors. This suggests that these mice indeed have a genetic instability and supports the possibility that they have an increased risk of additional genetic alterations in the remaining ptc allele.

To elucidate the molecular mechanisms underlying cancer predisposition in NBCCS patients, we have investigated cell cycle and apoptosis regulations using lymphoblastoid cells established from NBCCS patients.

MATERIALS AND METHODS

Cells and culture conditions Peripheral blood lymphocytes (PBLs) were obtained from three Japanese patients fulfilling the diagnostic criteria of NBCCS, after informed consent had been given. Two of the patients (patients 1 and 2) were 14- and 17-year-old sisters and presented multiple nevi, pits on palms and soles, jaw cysts, falx calcification and macrocephaly. Their father had similar clinical symptoms and tumors of maxilla. Patient 3, who was 59 years old, had multiple basal cell carcinomas, palmar pits and characteristic facial features, but no apparent family history of NBCCS. Three immortalized cell lines, namely G1, G2 and G3, were established from PBLs obtained from patients 1, 2 and 3, respectively, by infection with Epstein-Barr virus (EBV) obtained from B95-8 cells. K1 and K2 were cell lines established from PBLs of healthy donors and were used as controls. These cells were cultured in RPMI 1640 medium supplemented with 20% fetal bovine serum, 50 U/ml penicillin and 0.1 mg/ml streptomycin, and incubated at 37°C in 5% CO₂ atmosphere. To expose cells to γ-rays, a Hitachi MBR-1520-A-TWZ irradiation apparatus (Hitachi, Tokyo) was used.

Mutation analysis A polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) approach was employed to detect PTCH mutations. Primers used to amplify each exon of PTCH were synthesized based on the previous report. Primers used to amplify each exon of PTCH were synthesized based on the previous report. PCR was performed using 100 ng of genomic DNA and Pfu DNA polymerase (Stratagene, La Jolla, CA) in PCR buffer containing 1.5 mM MgCl₂ and [α-32P]dATP for 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 3 min. Products were denatured at 94°C for 5 min and loaded on 5% non-denatured polyacrylamide gels. The following four running buffers were used: 0.5× TBE with or without 10% glycerol and 1.0× TBE with or without 10% glycerol. The running condition was either at 4°C or at room temperature. Thus we analyzed the products under 8 different conditions for SSCP. After 12–18 h at 200–300 V, gels were dried and exposed to an X-ray film for 6–24 h. PCR products showing an aberrant mobility compared to those of healthy donors were sequenced after subcloning on a plasmid using a DNA sequencing kit and an automatic DNA sequencer (373A, Applied Biosystems, Foster City, CA). Sequence variations were confirmed in at least 4 independent colo-

Fig. 1. Mutation analyses of NBCCS-derived cells. (a) PCR-SSCP profiles showing aberrant bands (indicated with arrows) detected in amplified products from exon 6 of G1 and G2. (b) Sequencing profiles obtained with an automatic sequencer showing a single nucleotide deletion at the consecutive 4 cytosine residues, in which the top of C corresponds to the 900 nucleotide position of the cDNA sequence. The identical mutation was detected in the G2 cells and the sibling patients (data not shown), but was not detected in more than 100 normal individuals.
Cell viability and cell cycle analysis Relative DNA content of cells before and after γ-irradiation was determined by flow cytometry. Cells were fixed on ice for 30 min in phosphate-buffered saline (PBS) containing 30% ethanol, and then incubated overnight at 4°C in PBS containing 0.1% Triton-X 100, 0.1 mM EDTA, and 50 µg/ml RNase A. Propidium iodide (PI) (50 µg/ml) was added just prior to analysis and cells were examined with a Becton-Dickinson FACSort (Franklin Lakes, NJ). The primary data were displayed as relative cell numbers versus fluorescence intensities, and the fraction of cells in each phase of the cell cycle was calculated with computer software, Modfit (Verity Software House, Topsham, ME). Cells having a reduced DNA content were regarded as apoptotic cells.

Immunoblot analysis Protein detection with western blotting was performed as described previously. Briefly, 30 µg of cell lysates was fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and electrophoretically transferred to a nitrocellulose membrane. Monoclonal antibodies against p53 (Pab 1801, Santa Cruz Biotechnology, Santa Cruz, CA), p21/Cip1 (Transduction Laboratories, Lexington, KY), p27/Kip1 (Transduction Laboratories), and retinoblastoma protein (Rb) (Pharminigen, San Diego, CA) were used as the primary antibody. Horseradish peroxidase-conjugated rabbit anti-mouse IgG (DAKO, Carpinteria, CA) was used as the secondary antibody and proteins were visualized by enhanced chemiluminescence (ECL) (Amersham, Uppsala, Sweden). Ponceau S staining of a part of each membrane confirmed that the same amount of protein was loaded in all tracks (data not shown).

RESULTS

Mutation analysis We screened all the exons of the PTCH gene for mutation by the PCR-SSCP method with DNA isolated from the three established cell lines. An abnormal pattern showing heterozygous mutation was detected by SSCP in exon 6 of the G1 and G2 cells, and a one-base deletion in consecutive 4 cytosine residues was identified by sequencing (Fig. 1, a and b). The top of the consecutive 4 cytosine residues corresponds to the 900 nucleotide position of the cDNA form (accession number U43148) occurring in the first large extracellular loop, and the mutation is predicted to cause a frameshift in translation, generating a stop codon 18 a.a. downstream of the mutation point. The mutation was confirmed in genomic DNA of patients 1 and 2, who are siblings. Although the mutation was not experimentally confirmed in their parents, it may have been transferred from their father, based on the family history. In contrast, no other abnormalities in the SSCP pattern were detected despite extensive efforts.
and no PTCH mutations appeared to be associated with patient 3.

**Cell cycle regulation** We then investigated the cell cycle regulation in the NBCCS cells after exposure to \( \gamma \)-irradiation, by flow cytometry. Irradiated cells of the three established NBCCS lines showed marked accumulation in the G2M phase of the cell cycle, while normal cells accumulated in both the G0G1 and G2M phases (Fig. 2a). The ratio of G2M/G0G1 was slightly high in the NBCCS cells even without irradiation, but increased to a range from 1.40 to 1.73 in NBCCS cells, whereas it was 0.440 in normal cells after irradiation (Table I). Fractions of apoptotic cells after irradiation were also significantly lower in NBCCS cells than in normal cells (3–7% versus 13–16%) (Fig. 2b). Similar results were also obtained when apoptotic cells were defined in terms of nuclear apoptotic morphology.

### Table I. Fractionations of Cells

| Cells | Phenotype | G0G1 | G2M | S | G2M/G0G1 ratio |
|-------|-----------|------|-----|---|---------------|
| **Without \( \gamma \)-irradiation** | | | | | |
| K1 Normal | 67.8±3.76 | 12.8±1.62 | 19.4±2.19 | 0.182 |
| G1 NBCCS | 54.4±37.9 | 13.7±4.73 | 31.9±8.40 | 0.252 |
| G2 NBCCS | 51.3±7.30 | 11.5±0.93 | 37.2±7.51 | 0.224 |
| G3 NBCCS | 52.5±12.6 | 13.3±3.00 | 34.3±10.1 | 0.253 |

| **48 h after 5 Gy \( \gamma \)-irradiation** | | | | | |
| K1 Normal | 70.03±10.18 | 15.1±11.40 | 11.9±9.14 | 0.21 |
| G1 NBCCS | 32.4±5.30*** | 55.9±4.00*** | 11.7±1.29 | 1.73 |
| G2 NBCCS | 32.7±5.28*** | 52.1±9.81*** | 15.2±4.56 | 1.59 |
| G3 NBCCS | 33.7±5.27*** | 47.2±8.33*** | 19.2±8.90 | 1.40 |

*Fractions are indicated as percentage values of the mean±SD from at least 3 independent experiments.

*b* Nevoid basal cell carcinoma syndrome.

*** \( P<0.01, \) ** \( P<0.02 \) (vs. K1 by \( t \) test).

![Fig. 3. Expression patterns of p27, p53, p21 and Rb after \( \gamma \)-irradiation. Cell lysates were obtained from K1 and G2 at the indicated time points after irradiation (5 Gy) and then subjected to immunoblotting with the indicated antibody. The expression levels of p27 markedly decreased in the G2 cells but not in the K1 cells after irradiation, while the expression profiles of the others did not differ between the two cell lines. Results with G1 and G3 cells were similar to those with G2, and results with K2 were almost the same as those with K1.](image-url)
involved in the G0G1 cell cycle arrest is p27/Kip1, which phosphorylate Rb, is inactivated by p21. Another molecule with previous findings that Cdks, having a function to expressed at the highest level (Fig. 3d). This is consistent and NBCCS cells, the Rb protein was transiently hypo-
tion factor, E2F, that otherwise transactivates several genes
form of the Rb protein binds to and inactivates a transcrip-
tion regulation. A dephosphorylated (not the phosphorylated)
retinoblastoma, and is known to be involved in cell cycle
control and apoptosis.
Expression of proteins involved in the cell cycle control
To understand the molecular mechanisms underlying the
Cell Cycle dysregulation, we analyzed the expression profiles
of several cell cycle-related proteins by immunoblotting
with specific antibodies. It has been well documented
that γ-irradiation causes accumulation of the p53 protein,
which leads to upregulation of p21/waf-1, a major inhibi-
tor of the cyclin/Cdk/PCNA complex, resulting in the
G2M1 cell cycle arrest. Both in the normal and NBCCS
cells, p53 was transiently accumulated by 3 h after irradia-
tion and then gradually decreased over 48 h (Fig. 3b).
Upregulation of p21 followed the p53 upregulation and
reached the maximum level at 24 h after irradiation (Fig. 3c).
Thus, no significant differences in the p53 and p21
expression patterns were observed between the normal and
NBCCS cells. The Rb gene is a tumor suppressor gene for
retinoblastoma, and is known to be involved in cell cycle
regulation. A dephosphorylated (not the phosphorylated)
form of the Rb protein binds to and inactivates a transcrip-
tion factor, E2F, that otherwise transactivates several genes
responsible for cell cycle progression. In both the normal
and NBCCS cells, the Rb protein was transiently hypo-
phosphorylated 24 h after irradiation when p21 was
expressed at the highest level (Fig. 3d). This is consistent
with previous findings that Cdks, having a function to
phosphorylate Rb, is inactivated by p21. Another molecule
involved in the G2M1 cell cycle arrest is p27/Kip1, which
is also an inhibitor of cyclin/Cdk.22, 23 We detected a sig-
ificant downregulation of p27 expression from 3 to 48 h
after irradiation in NBCCS cells, while almost no change,
or even a slight increase, was observed in normal cells
(Fig. 3a). Densitometric analyses showed a decrease to
49–62% of the original amount in the G1 and G2 cells
after 5 or 10 Gy irradiation.

DISCUSSION
We have identified a germline mutation of the PTCH
gene in two cell lines, G1 and G2, which were derived
from sibling patients. The mutation caused a premature
termination in translation, and the cells seemed to produce
a half amount of the intact PTCH protein. In contrast, no
PTCH mutations were detected in established cells or in
genomic DNA of patient 3. It is well known that a small
mutation, such as a single nucleotide substitution, is some-
times difficult to detect with SSCP. Therefore, it is still
possible that patient 3 does indeed carry a mutation in the
PTCH gene. Alternatively, a mutation may exist in the
areas that we have not analyzed, for example, in a pro-
moter or intron region. Previous reports by several groups
revealed a PTCH mutation in only 33% of NBCCS
patients,10, 11 which is relatively low compared to usual
results in mutational analyses for other diseases. In this
regard, it is intriguing that activating mutations of the
SMO gene, whose product is inactivated by the PTCH
protein, were detected in sporadic cases of basal cell
carcinoma.24, 25 Therefore, SMO may be another so-far-
unidentified target for germline mutations in NBCCS.

Despite mutation detection, all cells of the three estab-
lished cell lines showed similar unusual features of cell
cycle control and apoptosis after γ-irradiation. NBCCS
cells were arrested in the G2M phase and normal cells in
the G2M1 phases after irradiation. The fraction of
apoptotic cells was smaller in NBCCS than in normal cells
after irradiation. The observed dysregulation of the cell
cycle and apoptosis is reminiscent of the Li-Fraumeni syn-
drome and ataxia telangiectasia (ATM), which also predis-
pose to cancer. In these disorders, the dysregulation is
observed in cells heterozygous of the respective responsi-
ble genes, p53 and ATM.26, 27 The cell cycle dysregulation
in Li-Fraumeni and ATM cells may be accounted for by
defects of the p53-p21 pathway, since the p53 protein is
the primary or immediate target for the diseases, respec-
tively. In NBCCS, however, expression of both p53 and
p21 after irradiation did not differ from that in normal
cells, and the p53-p21 pathway seemed to be intact.
According to a report on mice lacking p21, DNA-damage-
induced G2M1 arrest is partially dependent on p21.28 This
implies that other genes are also involved in cell cycle
arrest. In this regard, it is interesting that p27/Kip1,
another cyclin/Cdk inhibitor, was significantly downregu-
lated after irradiation of NBCCS cells. In Drosophila,
SHH induces expression of the decapentaplegic gene,
which is related to mammalian transforming growth factor
β (TGF-β).29 TGF-β can induce G2M cell cycle arrest
through activation of p27.22 Although p27 is reported to
be dispensable for G1 arrest in embryonic fibroblasts,30
the role of p27 may be cell type-dependent and an irradi-
ation-mediated decrease in p27 levels may explain the rela-
tive defect in G1 arrest and predominant arrest in G2M
after γ-irradiation of NBCCS lymphoblasts. On the other
hand, γ-irradiation-induced signaling leading to G2M arrest
was intact in NBCCS cells. This implies that molecules
responsible for the G1 checkpoint, such as 14-3-3σ, Chk1
and Cdc25C, are not affected in this disorder.31–33

Finally, several groups have reported recently that
expression levels of p27 in cancer cells are inversely cor-
related with the prognosis.34, 35 In this regard, decreased
levels of p27 expression after γ-irradiation may explain the
cancer predisposition of NBCCS patients.
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REFERENCES

1) Gorlin, R. J. and Goltz, R. W.  Multiple nevoid basal-cell epithelioma, jaw cysts and bifid rib. A syndrome.  *N. Engl. J. Med.*, 262, 908–912 (1960).

2) Evans, D. G., Ladusans, E. J., Rimmer, S., Burnell, L. D., Thakker, N. and Fardon, P. A.  Complication of the nevoid basal cell carcinoma syndrome: results of a population based study.  *J. Med. Genet.*, 30, 460–464 (1993).

3) Gorlin, R. J.  Nevoid basal cell carcinoma syndrome.  *Dermatol. Clin.*, 13, 113–125 (1995).

4) Springate, J. E.  The nevoid basal cell carcinoma syndrome.  *J. Pediatr. Surg.*, 21, 908–910 (1986).

5) Evans, D. G., Fardon, P. A., Burnell, L. D., Gattamaneni, H. R. and Birch, J. M.  The incidence of Gorlin syndrome in 173 consecutive cases of medulloblastoma.  *Br. J. Cancer*, 64, 959–961 (1991).

6) Gorlin, R. J.  Nevoid basal-cell carcinoma syndrome.  *Medicine*, 66, 98–113 (1987).

7) Atahan, I. L., Yildiz, F., Ozyar, E., Uzal, D. and Zorlu, F.  Basal cell carcinomas developing in a case of medulloblastoma associated with Gorlin’s syndrome.  *Pediatr. Hematol. Oncol.*, 15, 187–191 (1998).

8) Hahn, H., Wicking, C., Zaphiropoulous, P. G., Gailani, M. R., Shanley, S., Chidambaram, A., Vorechovsky, I., Holmberg, E., Unden, A. B., Gillies, S., Negus, K., Smyth, I., Pressman, C., Leffell, D. J., Gerrard, B., Goldstein, A. M., Dean, M., Toftgard, R., Chenevix-Trench, G., Wainwright, B. and Bale, A. E.  Mutations of the human homolog of *Drosophila* patched in the nevoid basal cell carcinoma syndrome.  *Cell*, 85, 841–851 (1996).

9) Johnson, R. L., Rothman, A. L., Xie, J., Goodrich, L. V., Bare, J. W., Bonifas, J. M., Quinn, A. G., Myers, R. M., Cox, D. R., Epstein, E. H., Jr. and Scott, M. P.  Human homolog of patched, a candidate gene for the basal cell nevus syndrome.  *Science*, 272, 1668–1671 (1996).

10) Chidambaram, A., Goldstein, A. M., Gailani, M. R., Gerrard, B., Bale, S. I., DiGiovanna, J. J., Bale, A. E. and Dean, M.  Mutations in the human homologue of the *Drosophila* patched gene in Caucasian and African-American nevoid basal cell carcinoma syndrome patients.  *Cancer Res.*, 56, 4599–4601 (1996).

11) Gailani, M. R., Stable-Backdahl, M., Leffell, D. J., Glynn, M., Zaphiropoulos, P. G., Pressman, C., Unden, A. B., Dean, M., Brash, D. E., Bale, A. E. and Toftgard, R.  The role of the human homologue of *Drosophila* patched in sporadic basal cell carcinomas.  *Nat. Genet.*, 14, 78–81 (1996).

12) Wicking, C., Shanley, S., Smyth, I., Gillies, S., Negus, K., Graham, S., Suthers, G., Haites, N., Edwards, M., Wainwright, B. and Chenevix-Trench, G.  Most germ-line mutations in the nevoid basal cell carcinoma syndrome lead to a premature termination of the PATCHED protein, and no genotype-phenotype correlations are evident.  *Am. J. Hum. Genet.*, 60, 21–26 (1997).

13) Marigo, V., Davey, R. A., Zuo, Y., Cunningham, J. M. and Tabin, C. J.  Biochemical evidence that patched is the hedgehog receptor.  *Nature*, 384, 176–179 (1996).

14) Arlett, C. F. and Priestley, A.  Deficient recovery from potentially lethal damage in some gamma-irradiated human fibroblast cell strains.  *Br. J. Cancer Suppl.*, 6, 227–232 (1984).

15) Featherstone, T., Taylor, A. M. and Harnden, D. G.  Studies on the radiosensitivity of cells from patients with basal cell naevus syndrome.  *Am. J. Hum. Genet.*, 35, 58–66 (1983).

16) Applegate, L. A., Goldberg, L. H., Ley, R. D. and Ananthaswamy, H. N.  Hypersensitivity of skin fibroblasts from basal cell nevus syndrome patients to killing by ultraviolet B but not by ultraviolet C radiation.  *Cancer Res.*, 50, 637–641 (1990).

17) Bassukus, I. D., Schell, H., Arai, A. and Hofmann, P.  Hyposensitivity of basal cell naevus syndrome dermal fibroblasts to ultraviolet A.  *Lancet*, 336, 8718 (1990).

18) Fuji, K., Suzuki, N., Ishijima, S., Kita, K., Sonoda, T., Dezawa, M., Sugita, K. and Niimi, H.  Abnormal DNA synthesis activity induced by X-rays in nevoid basal cell carcinoma syndrome cells.  *Biochem. Biophys. Res. Commun.*, 240, 269–272 (1997).

19) Hahn, H., Wojnowski, L., Zimmer, A. M., Hall, J., Miller, G. and Zimmer, A.  Rhabdomyosarcomas and radiation hypersensitivity in a mouse model of Gorlin syndrome.  *Nat. Med.*, 4, 619–622 (1998).

20) Kimonis, V. E., Goldstein, A. M., Pastakia, B., Yang, M. L., Kase, R., DiGiovanna, J. J., Bale, A. E. and Bale, S. J.  Clinical manifestations in 105 persons with nevoid basal cell carcinoma syndrome.  *Am. J. Med. Genet.*, 69, 299–308 (1997).

21) Miyashita, T., Krajewski, S., Krajewska, M., Wang, H. G., Lin, H. K., Liebermann, D. A., Hoffman, B. and Reed, J. C.  Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression *in vitro* and *in vivo*.  *Oncoogene*, 9, 1799–1805 (1994).

22) Polyak, K., Lee, M. H., Erdjument-Bromage, H., Koff, A., Roberts, J. M., Tempst, P. and Massague, J.  Cloning of p27Kip1, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals.  *Cell*, 78, 59–66 (1994).
23) Toyoshima, H. and Hunter, T. p27, a novel inhibitor of G1 cyclin-Cdk protein kinase activity, is related to p21. *Cell*, 78, 67–74 (1994).

24) Xie, J., Murone, M., Luo, S. M., Ryan, A., Gu, Q., Zhang, C., Bonifas, J. M., Lam, C. W., Hynes, M., Goddard, A., Rosenthal, A., Epstein, E. H., Jr. and de Sauvage, F. J. Activating smoothened mutations in sporadic basal-cell carcinoma. *Nature*, 391, 90–92 (1998).

25) Reifenberger, J., Wolter, M., Weber, R. G., Megahed, M., Ruzicka, T., Lichter, P. and Reifenberger, G. Missense mutations in SMOH in sporadic basal cell carcinomas of the skin and primitive neuroectodermal tumors of the central nervous system. *Cancer Res.*, 58, 1798–1803 (1998).

26) Goi, K., Takagi, M., Iwata, S., Delia, D., Asada, M., Donghi, R., Tsunematsu, Y., Nakazawa, S., Yamamoto, H., Yokota, J., Tamura, K., Sae, K., Utsunomiya, J., Takahashi, T., Ueda, R., Ishioka, C., Eguchi, M., Kamata, N. and Mizutani, S. DNA damage-associated dysregulation of the cell cycle and apoptosis control in cells with germ-line p53 mutation. *Cancer Res.*, 57, 1895–1902 (1997).

27) Takagi, M., Delia, D., Chessa, L., Iwata, S., Shigeta, T., Kanke, Y., Goi, K., Asada, M., Eguchi, M., Kodama, C. and Mizutani, S. Defective control of apoptosis, radiosensitivity, and spindle checkpoint in ataxia telangiectasia. *Cancer Res.*, 58, 4923–4929 (1998).

28) Deng, C., Zhang, F., Harper, J. W., Elledge, S. J. and Leder, P. Mice lacking p21<sup>Cip1/WAF1</sup> undergo normal development, but are defective in G1 checkpoint control. *Cell*, 82, 675–684 (1995).

29) Treisman, J. E., Lai, Z. C. and Rubin, G. M. Shortsighted acts in the decapentaplegic pathway in *Drosophila* eye development and has homology to a mouse TGF-beta-responsive gene. *Development*, 121, 2835–2845 (1995).

30) Nakayama, K., Ishida, N., Shirane, M., Inomata, A., Inoue, T., Shishido, N., Horii, I., Loh, D. Y. and Nakayama, K. Mice lacking p27<sup>Kip1</sup> display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. *Cell*, 85, 707–720 (1996).

31) Sanchez, Y., Wong, C., Thoma, R. S., Richman, R., Wu, Z., Piwnica-Worms, H. and Elledge, S. J. Conservation of the Chk1 checkpoint pathway in mammals: linkage of DNA damage to Cdk regulation through Cdc25. *Science*, 277, 1497–1501 (1997).

32) Peng, C. Y., Graves, P. R., Thomas, R. S., Wu, Z., Shaw, A. S. and Piwnica-Worms, H. Mitotic and G2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216. *Science*, 277, 1501–1505 (1997).

33) Hermeking, H., Lengauer, C., Polyak, K., He, T.-C., Zhang, L., Thiagalingam, S., Kinzler, K. W. and Vogelstein, B. 14-3-3σ is a p53-regulated inhibitor of G2/M progression. *Mol. Cell.*, 1, 3–11 (1997).

34) Lloyd, R. V., Erickson, L. A., Jin, L., Kulig, E., Qian, X., Cheville, J. C. and Scheithauer, B. W. p27kip1: a multifunctional cyclin-dependent kinase inhibitor with prognostic significance in human cancers. *Am. J. Pathol.*, 154, 313–323 (1999).

35) Catzavelos, C., Tsao, M. S., DeBoer, G., Bhattacharya, N., Shepherd, F. A. and Slingerland, J. M. Reduced expression of the cell cycle inhibitor p27Kip1 in non-small cell lung carcinoma: a prognostic factor independent of Ras. *Cancer Res.*, 59, 684–688 (1999).