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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 G_2M phase after γ-irradiation, whereas normal cells showed cell cycle arrest both in the G_0G_1 and G_2M 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 and vertebrate anomalies and also an increased risk for tumor development, including basal cell carcinomas, medulloblastomas, ovarian and cardiac fibromas, meningiomas, fibrosarcomas, and rhabdomyosarcomas.[2, 3)] In particular, it is well known that 1–2% of medulloblastomas and 0.5% of basal cell carcinomas of sporadic origins are attributable to this syndrome.[4, 5)] Another clinical feature of NBCCS patients is abnormal sensitivity to radiotherapeutic doses of γ-irradiation.[6)] Several NBCCS patients with medulloblastomas treated by radiation therapy have been reported to develop a large number of basal cell carcinomas in the irradiated skin area in a short period after irradiation.[7)]

A human homologue (*PTCH*) of the *Drosophila* segment polarity gene, patched (*ptc*), has been identified as the gene responsible for NBCCS by mapping of affected families and analysis of loss-of-heterozygosity in tumors followed by positional cloning.[8, 9)] The human *PTCH* gene 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.[8)] 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.[8–12)] This suggests that a reduction in expression of the *PTCH* gene leads to the developmental abnormalities observed in the syndrome and that complete loss of the patched function contributes to tumor formation.[8)]

The *Drosophila ptc* gene has been shown to function in the hedgehog signal pathway to form the anterio-posterior 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.

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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.[14–17] 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,[18] 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.[19] 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.[19]

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,[20] 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% CO_2 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.[8, 10] PCR was performed using 100 ng of genomic DNA and Pfu DNA polymerase (Stratagene, La Jolla, CA) in PCR buffer containing 1.5 m*M* $MgCl_2$ and [α-^{32}P]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-

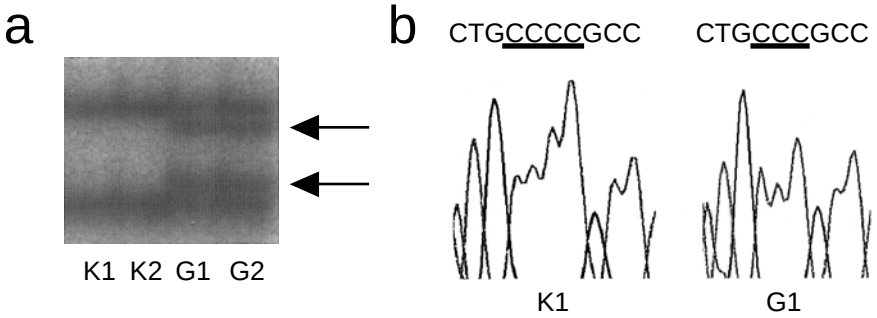

Fig. 1. Mutation analyses of NBCCS-derived cells. (a) PCR-SSCP profiles showing aberrant bands (indicated with arrows) detected in amplified products from exon 6 of G1 and G2. (b) Sequencing profiles obtained with an automatic sequencer showing a single nucleotide deletion at the consecutive 4 cytosine 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.

nies, and also by means of direct sequencing of the amplified products.

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 m*M* 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.[21] 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) (Pharmingen, 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,

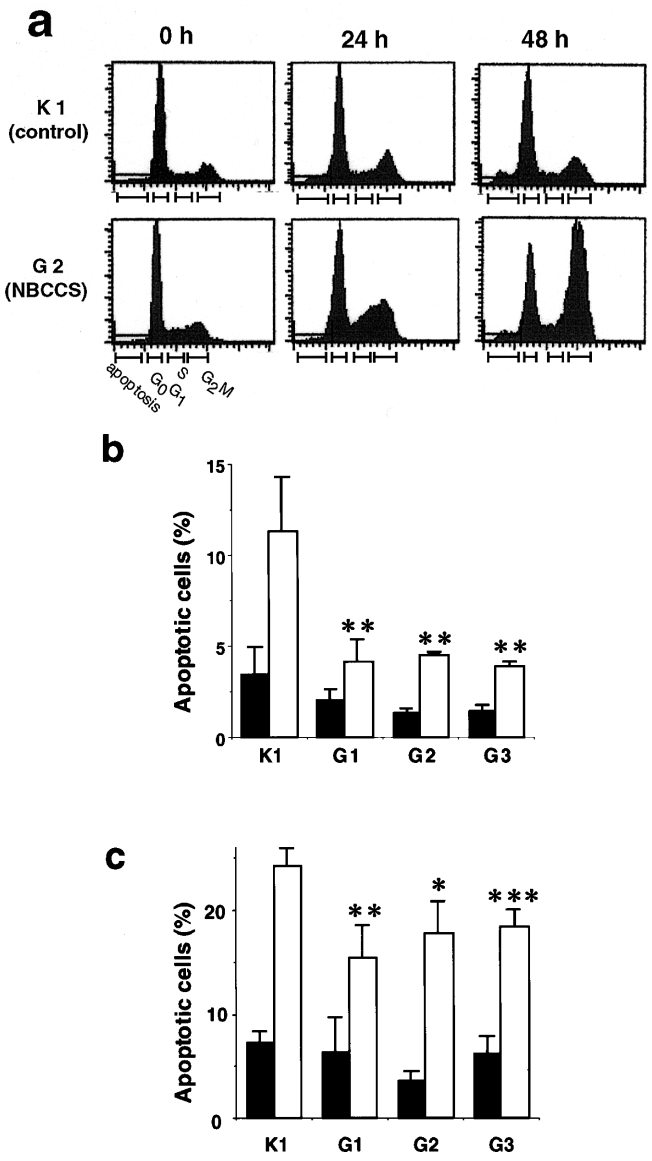

Fig. 2. Flow cytometric analysis of γ-irradiated cells, indicating dysregulation of cell cycle control in NBCCS cells. (a) Flow cytometric profiles for DNA contents of K1 and G2 cells after γ-irradiation. K1 and G2 cells were irradiated with γ-rays (5 Gy) and subjected to analysis at the indicated time points. The profiles are displayed as fluorescence intensities of PI versus relative cell numbers. Cells were fractionated into four classes, apoptosis, G_0G_1, S and G_2M, with the indicated gating. Results with G1 and G3 cells were similar to those with G2, and results with K2 were the same as those with K1 (see also Table I). (b) Fraction of apoptotic cell population before (filled column) and after (open column) γ-irradiation, indicating a decrease of the apoptotic fraction in NBCCS cells after irradiation. (c) The same experiment as in (b) was performed and apoptotic cells were counted on the basis of nuclear morphology after staining with Hoechst 33342. At least 200 cells were counted for each condition. Mean values±SD from at least 3 independent experiments are presented. *** $P<0.01$, ** $P<0.02$, * $P<0.05$ (vs. K1 by *t* test).

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 γ-irradiation, by flow cytometry. Irradiated cells of the three established NBCCS lines showed marked accumulation in the G_2M phase of the cell cycle, while normal cells accumulated in both the G_0G_1 and G_2M phases (Fig. 2a). The ratio of G_2M/G_0G_1 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	G_0G_1	G_2M	S	G_2M/G_0G_1 ratio
Without γ-irradiation					
K1	Normal	67.8±3.76[a)]	12.8±1.62	19.4±2.19	0.182
G1	NBCCS[b)]	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 γ-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

a) 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).

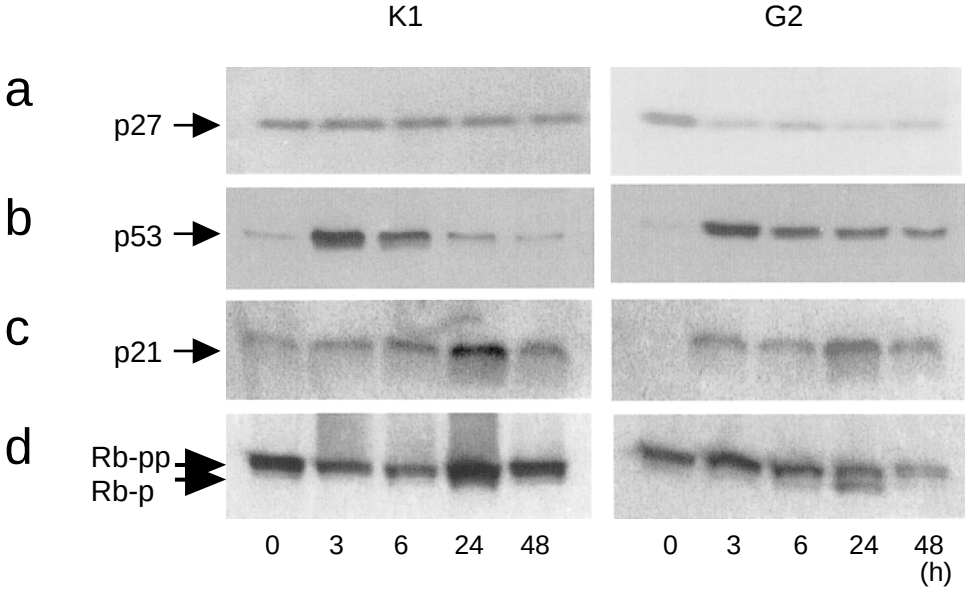

Fig. 3. Expression patterns of p27, p53, p21 and Rb after γ-irradiation. Cell lysates were obtained from K1 and G2 at the indicated time points after irradiation (5 Gy) and then subjected to immunoblotting with the indicated antibody. The expression levels of p27 markedly decreased in the G2 cells but 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.

(Fig. 2c). Increased cell cycle arrest in the G_2M phase and a decrease of apototic cells were also observed after irradiation with a different dose of 10 Gy (data not shown). These results clearly show that NBCCS cells are distinctive 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 inhibitor of the cyclin/Cdk/PCNA complex, resulting in the G_0G_1 cell cycle arrest. Both in the normal and NBCCS cells, p53 was transiently accumulated by 3 h after irradiation 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 transcription factor, E2F, that otherwise transactivates several genes responsible for cell cycle progression. In both the normal and NBCCS cells, the Rb protein was transiently hypophosphorylated 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 G_0G_1 cell cycle arrest is p27/Kip1, which is also an inhibitor of cyclin/Cdk.[22, 23] We detected a significant 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 sometimes 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 promoter 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 established cell lines showed similar unusual features of cell cycle control and apoptosis after γ-irradiation. NBCCS cells were arrested in the G_2M phase and normal cells in the G_0G_1 and G_2M 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 syndrome and ataxia telangiectasia (ATM), which also predispose to cancer. In these disorders, the dysregulation is observed in cells heterozygous of the respective responsible 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, respectively. 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 G_0G_1 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 downregulated 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 G_0G_1 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 irradiation-mediated decrease in p27 levels may explain the relative defect in G1 arrest and predominant arrest in G_2M after γ-irradiation of NBCCS lymphoblasts. On the other hand, γ-irradiation-induced signaling leading to G_2M arrest was intact in NBCCS cells. This implies that molecules responsible for the G_2 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 correlated 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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