Establishment of Immortal Normal and Ataxia Telangiectasia Fibroblast Cell Lines by Introduction of the \textit{hTERT} Gene

HIDEAKI NAKAMURA, HIROKO FUKAMI, YUKO HAYASHI, TOHRU KIYONO, SHIGEKAZU NAKATSUGAWA, MICHINARI HAMAGUCHI, and KANJI ISHIZAKI

*Corresponding author: Phone: +81–52–762–6111 (ext. 7012), Fax: +81–52–763–5233, E-mail: hnakamu@aichi-cc.jp

1 Central Laboratory and Radiation Biology, Aichi Cancer Center Research Institute, Kanokoden 1–1, Chikusa-ku, Nagoya 464–8681, Japan
2 Department of Molecular Pathogenesis, Nagoya University School of Medicine, Tsurumai-cho 65, Showa-ku, Nagoya 466–8550, Japan
3 Division of Virology, Aichi Cancer Center Research Institute, Kanokoden 1–1, Chikusa-ku, Nagoya 464–8681, Japan
4 Department of Radiology, Nagoya University School of Medicine, Tsurumai-cho 65, Showa-ku, Nagoya 466–8550, Japan

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To establish immortal human cells, we introduced the \textit{human catalytic subunit of telomerase (hTERT)} gene into skin fibroblast cells obtained from normal and ataxia telangiectasia (AT) individuals of Japanese origin. After \textit{hTERT} introduction, these cells continue to grow beyond a population doubling number of 200 while maintaining their original radiosensitivity. Inductions of p53, phosphorylation of Ser15 in p53, and induction of p21 by X-ray irradiation in immortal cells derived from normal individual were not affected by the \textit{hTERT} introduction. Both normal and AT immortal cells exhibited an apparent inhibition of growth as original primary cells when they reached confluence. Karyotype analysis has revealed that they are in a diploid range. These results suggest that cells immortalized by \textit{hTERT} introduction retain their original characteristics except for immortalization, and that they may be useful for analyzing various effects of radiation on human cells.

INTRODUCTION

High-dose radiation frequently leads to the loss of essential cellular functions and/or to irreparable chromosome damage, resulting in cell death, whereas low-dose radiation chiefly induces rare and barely detectable genetic changes, mainly within chromosomal regions non-essential for cell survival\(^1\). Several studies on mammalian cells demonstrated that low-dose rate radiation (LDR) caused a significant reduction in mutagenic effectiveness\(^2–4\). Thus, it may be important to know the effects of LDR on human cells in order to assess the effects of radiation from nuclear power plants and space flights\(^5\).

In analyzing the genetic effects of LDR on human cells, the adaptive response to radiation may be an important factor. It is also becoming clear that this response is mediated by cellular signal transduction mechanisms, which are mainly involved with the \textit{p53} gene and others\(^6\). These suggest that the use of SV40-immortalized cells\(^7\) or cancer-derived cells may not be appropriate for analyzing the effect of LDR on human cells. However, since diploid human cells have a limited life span and reach senescence after a certain number of cell divisions, they are also not suitable for this purpose\(^8\).

Recently, it was shown that the introduction and forced expression of the \textit{human catalytic subunit of telomerase (hTERT)} gene can extend the life span of
human cells without any change in the fundamental cellular characteristics. Cells introduced with hTERT exhibited normal cell cycle controls, functional p53 and RB checkpoints, anchorage-dependent contact inhibition, and growth-factor-dependent proliferation. In addition, these cells possess a normal karyotype. These results indicate that these cells may be suitable materials to analyze the effects of LDR on human cells, and also for large-scale biochemical and molecular experiments such as gene transfer or gene inactivation.

Human population exhibits a large genetic variation like a single nucleotide polymorphism (SNP), and some of such genetic variations may be significantly related to an individual’s response to various environmental factors. It is reported that SNP distribution and allele frequency in a Japanese population differ from those of other ethnic groups. Therefore, to assess the genetic effects of LDR on Japanese people, cells obtained from Japanese individuals are more preferable. However, so far reported cells immortalized by hTERT were not derived from Japanese origin.

In this study, we introduced the hTERT gene and have established immortalized cell lines derived from a normal individual and AT patients of Japanese origin. We also analyzed the response to radiation of these immortal cells to make sure that these cells are useable in a future study analyzing the genetic effects of LDR on human cells.

MATERIALS AND METHODS

Cell culture and gene transfer

Skin fibroblasts from a Japanese normal individual (SuSa) and Japanese Ataxia telangiectasia patients (AT1OS, AT1KY and AT2KY), and hTERT-transfected cells were cultured in DMEM medium (Sigma)

H.I. AT2KY/T-n, PDN 29 SuSa/T-n, PDN 86 AT1KY/T-n, PDN 32 AT1KY, PDN 6 AT1OS/T-n, PDN 109

Chaps TSR 8

Fig. 1. Telomerase activities in cells transfected with vector alone and hTERT gene were detected by TRAP assay. Chaps buffer without cell extract and heat inactivation of cell extracts (H.I. +) was used as negative control. TSR 8 was a positive control supplied in the assay kit.
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Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (EQUI TECH-BIO INC., Ker rville, TX).

An EcoRI-BamHI hTERT cDNA fragment containing the Kozak consensus sequence and the coding sequence of hTERT franked by the restriction sites were obtained by PCR with pLXSN-hTERT (a kind gift from Dr. Denis A. Galloway (FHCRC, USA)) as a template. The retrovirus vector pCLXSN-hTERT was constructed by inserting the hTERT EcoRI-BamHI fragment between the EcoRI and BamHI sites of pCLXSN20 (Imgenex Corp., San Diego, CA). Preparation of hTERT- and LXSN-retroviruses and infection protocols have been described previously20. After transfection of these retrovirus-vectors, G418-resistant cells were selected and continuously cultured with subcultures once a week. The cells introduced with the hTERT gene were identified by adding “T-n” behind their original names (e.g., SuSa/T-n), and cells trans-

![Fig. 2. Analysis of terminal restriction fragments (TRF) length in original and hTERT-transfected cells. Telomere lengths were longer in hTERT-transfected cells than in original cells used in PDN 4–6.](image)

![Fig. 3. Growth curves of control and hTERT-introduced cells. All hTERT-introduced cells have grown far beyond the points at which control cells stopped growing. AT1OS/neo cells were lost in transfection of the neo-vector because of contamination.](image)
fected with vector alone were identified by adding “neo.”

**Telomerase activity**

Telomerase activity in each cell line was detected by the telomeric repeat amplification protocol (TRAP) assay using the TRAPEze kit (Intergen, Purchase, NY) according to the manufacturer’s protocol.

**Telomere length analysis**

Terminal restriction fragment (TRF) length was determined by southern blotting. Total genomic DNA was isolated from each cell line by proteinase K digestion. Ten µg of genomic DNA was digested with Hinfl and RsaI for 16 hr, separated on a 0.7% agarose gel, and transferred onto a nylon membrane. TRF was detected by hybridization with a telomeric probe (CCCTAA)₄, end-labeled with [γ⁻³²P]ATP.

**Analysis of contact inhibition**

To determine whether immortal cells still retain the ability for contact inhibition, cells plated on a coverslip were cultured to confluence, and then 10 mM of bromodeoxyuridine (BrdU) was added to the medium. After 24 hr at 37°C, the cells which incorporated BrdU were stained with FITC-labeled anti-BrdU antibody (Neo Markers, Fremont, CA) and observed under a fluorescence microscope.

**Karyotype analysis**

Fixation and chromosome preparation were performed according to the standard procedure described previously. For each cell line more than 50 cells were scored for their chromosome number.

**Cellular sensitivity to x-ray irradiation**

The appropriate number of cells inoculated into a 6-cm dish were irradiated with X-ray, using a model MBR1520R X-ray machine (Hitachi Medico, Tokyo) at a dose rate of 2 Gy/min, up to 8 Gy (for SuSa and SuSa/T-n) or 3 Gy (for AT1OS and AT1OS/T-n). After irradiation, the cells were incubated for 14 days and stained with crystal violet. Only colonies containing >50 cells were scored.

**Western blot analysis**

To observe the induction of p53 protein, phosphorylation of p53 protein at Ser15, and induction of p21 protein, SuSa and SuSa/T-n cells were irradiated with 10 Gy X-ray during the growing stage, and those proteins were extracted with RIPA solution (1% NP-40, 1% sodium deoxycholate and 0.05% SDS in PBS) at various times after X-irradiation. Ten µg of total protein was loaded onto a 10% SDS gel and blotted onto a nitrocellulose membrane after electrophoresis. The primary mouse monoclonal anti-p53 (Santa Cruz

### Table 1. Detection of cells in the S-phase during 24 hr by BrdU incorporation

| Cell              | BrdU-labelled cells | Total cells | Labelling index (%) |
|-------------------|---------------------|-------------|---------------------|
| SuSa/T-n, PDN60  | 138                 | 2008        | 6.9                 |
| SuSa/T-n, PDN60  | 1161                | 1813        | 64.0                |
| AT1OS/T-n, PDN80 | 92                  | 1204        | 7.6                 |
| AT1OS/T-n, PDN80 | 224                 | 510         | 43.9                |

Immortalized cells were cultured in a medium with BrdU for 24 hr.

![Fig. 4.](https://example.com/4.png) Chromosome numbers in immortalized cells. Both immortalized cells analyzed (SuSa/T-n at PDN 128, hatched bars; AT1OS/T-n at PDN 150, open bars) are in a diploid range with modal chromosome number of 46.
Biotechnology Inc., Santa Cruz, CA), rabbit polyclonal anti-Ser15-phosphorylated p53 (New England Biolabs Inc., Beverly, MA), and mouse monoclonal anti-p21 (NEOMARKERS, Fremont, CA) were used in a 1:200, 1:500 and 1:1000 dilution, respectively. The secondary antibodies were peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (Zymed Laboratories, Inc., San Francisco, CA,) used in a 1:10000 dilution. The Western Blot Chemiluminescence Reagent Plus (NEN™ Life Science Products Inc., Boston, MA) was then used for detection with X-ray films.

**RESULTS**

We transfected fibroblast cells obtained from normal individual and AT patients with hTERT gene. After transfection of the hTERT expression vector, stable transfectants were selected by G418. G418-resistant cells were then continuously subcultured more than 2 years.

Telomerase activity was not detected in the vector alone-transfected cells by the TRAP assay. In contrast, all of the cells transfected with the hTERT gene exhibited significant telomerase activity (Fig. 1). The telomere length determined by the TRF length analysis was longer in the hTERT-transfected cells than in the original cells (Fig. 2).

Although the cells transfected with vector alone reached complete senescence before the population doubling number (PDN) 50, all the cells transfected with the hTERT gene have continued to grow beyond PDN 200 with no indication of senescence like a flat shape and reduced growth rate (Fig. 3). Because SuSa/T-n and AT1OS/T-n cells showed better growth properties such as growth rate and colony forming efficiency

![Graph showing X-ray sensitivity](https://example.com/graph.png)

**Fig. 5.** X-ray sensitivity of original and immortalized cells. Original (PDN 4–6), SuSa/T-n (PDN 105), and AT1OS/T-n (PDN 127) cells were irradiated with the doses indicated for colony formation assay. Both immortalized cells showed close radiosensitivity to their original cells. Bars in figure indicate standard deviations (n = 3).
than other hTERT-transfected cells, we further characterize these two cell lines.

BrdU-uptake analysis clearly showed that immortal cells still exhibit apparent growth arrest after reaching confluency (Table 1). Karyotype analysis showed that both of these immortalized cells keep a diploid range with a modal number of 46 (Fig. 4).

The hTERT-immortalized cells and original cells were exposed to various doses of X-ray radiation for a colony-formation assay. Immortalized normal cells maintained their original radiosensitivity (Fig. 5). Among AT cells, AT1OS/T-n cells seemed to be slightly more resistant than the original AT1OS cells. However, the difference is very small, and AT1OS/T-n cells are still much more sensitive than SuSa cells.

We analyzed induction of p53, phosphorylation of Ser15 in p53, and induction of p21 using western blot analysis. p53 protein in SuSa and SuSa/T-n cells following 10 Gy X-ray exposures had increased in the first few hours, and then gradually decreased, indicating the typical p53 induction reported in other normal human cells. Phosphorylation of Ser15 of p53 was also induced by X-ray irradiation in both SuSa and SuSa/T-n cells in the same manner as indicated in previous reports. p21 protein, known to be induced by p53, was also similarly induced in both cells, suggesting that the p53 pathway was still active in SuSa/T-n cells.

DISCUSSION

The present study demonstrates that the expression of hTERT can immortalize skin fibroblasts derived from the normal individual and AT patients. This is consistent with the previous reports that the life span of human fibroblast cells was easily extended by introduction of the hTERT gene. However, it is reported that human fetal lung fibroblasts transfected with hTERT gene may undergo a crisis following an extended life span of PDN 160. In contrast, our hTERT-introduced cells continuously grow beyond PDN 200, and SuSa/T-n and AT1OS/T-n are still growing even beyond PDN 300, suggesting no senescence. These different results may be caused by the fact that fibroblast cells transfected with hTERT gene originated from different tissues.

We determined the characteristics of our hTERT-introduced cells, and the results are consistent with those in the previous reports, suggesting that hTERT-transfected fibroblasts are immortal, yet retain their original characteristics and repair activity. Cells with life span extended by hTERT were reportedly not tumorigenic when transplanted in mice. This lends support to the notion that our hTERT-immortalized cells are not transformed.

The result of western blot analysis suggests that SuSa/T-n cells retain the normal p53 response after

![Fig. 6. Western blot analysis of p53 protein, phosphorylation of p53 protein at serine 15, and induction of p21 protein by X-ray irradiation, in SuSa (PDN 6) and SuSa/T-n cells (PDN 74). Induction of each protein was determined 0, 1, 2, 3, 4 and 6 hr after exposure to 10 Gy X-ray. Clear induction was observed in both cells.](https://academic.oup.com/jrr/article-abstract/43/2/167/1049532)
DNA damage. Consistent with this finding, Wood et al. reported that the p53-dependent G1 checkpoint in response to ionizing radiation was intact in normal human fibroblasts expressing hTERT\textsuperscript{12}). In SuSa and SuSa/T-n cells, the induction of p53 and phosphorylated p53 protein essential for p53 activation after X-ray irradiation\textsuperscript{25}) appears to be similar, whereas induction of p21 protein in SuSa/T-n cells has a slightly different time course from that of the SuSa cells. This small difference may be caused by a difference in PDN of the cells used in this experiment.

Recently, p53 protein has been found to play a key role in the adaptive response, which may be very important in analysis of the biological effect of low-dose radiation\textsuperscript{26}). Since the SuSa/T-n cells exhibited a p53-signaling pathway, they may also have an adaptive response as original SuSa cells. Therefore, such immortalized cells would be preferable for analyzing the genetic effects of LDR and any other types of radiation on human cells, compared to other immortal human cells such as SV40-immortalized or cancer-derived cells.

For the genetic modification of cells such as gene transfer and gene inactivation, diploid cells may be inappropriate because they readily reach senescence in long-term experiments. However, with the present immortal cells, any kind of genetic engineering method developed in human tumor cells such as HeLa cells may be applicable. These immortal cells may also be used with shuttle vector systems developed to analyze radiation-induced mutations more precisely at the molecular level\textsuperscript{14,15}). The recently reported RNA-interference technique in mammalian cells seems to be effective in analyzing the function of cellular genes in response to radiation\textsuperscript{16}). Using the present immortal cells, this technique may also be applicable in future experiments.

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