J. Radiat. Res., 45, 105–110 (2004)

Regular Paper

Delayed Induction of Telomere Instability in Normal Human Fibroblast Cells by Ionizing Radiation

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Ionizing radiation/Genomic instability/Normal human cells/Telomere.

We examined the delayed induction of telomere instability in hTERT-immortalized normal human fibroblast (BJ1-hTERT) cells exposed to X-rays. BJ1-hTERT cells were irradiated with 2 Gy of X-rays, and chromosome aberrations were analyzed 24 hours after irradiation and in the surviving cells 14 days after X-ray exposure. We found that the X-ray-surviving cells showed an increased frequency of chromatid gaps and breaks and chromosome fragments compared to the control cells. Furthermore, centromere- and telomere-FISH revealed that the frequency of telomere loss and duplication significantly increased in surviving cells compared to the control level. Because no induction of telomere abnormality was observed in cells 24 hours after irradiation, X-irradiation might not affect telomeres directly, but it specifically induces delayed telomere instability in normal human fibroblast cells.

INTRODUCTION

Several recent studies have shown that various effects are induced in cells that are not directly irradiated, but that are the progeny of irradiated cells. These delayed effects are collectively termed radiation-induced genomic instability, which manifests itself as the appearance of delayed reproductive death, delayed chromosomal instability, and delayed mutagenesis.[1,2] Although DNA double-strand breaks have been reported to be involved in the initiation of radiation-induced genomic instability,[3,4] the mechanisms of perpetuation and the induction of delayed phenotypes have not yet been clarified.

Recently, we suggested that telomere instability is involved in delayed chromosomal instability induced in normal human diploid cells exposed to X-rays, because most of the dicentrics that formed more than 40 population doublings after irradiation were not accompanied by fragments.[5] It has been shown in several studies that dysfunctional shortened telomeres can induce end-to-end chromosome fusions, which produce dicentric chromosomes.[6,7] The dysfunction of telomeres initiates a breakage-fusion-bridge cycle (BFB cycle)[8,9] and may promote the perpetuation and induction of delayed chromosomal instability. More recently, telomere instability induced by a defect in DNA repair proteins, such as Ku86 or DNA-PKcs, or by over expression of dominant negative TRF2 causes chromosome aberration, indicating that telomere instability, which causes chromosome fusions,[10–12] could be one of the mechanisms of radiation-induced genomic instability. In rodent cells, interstitial telomere like repeats may be involved in delayed chromosomal instability.[13]

In the present study, we examined delayed telomere instability in X-ray-surviving normal human diploid cells immortalized by the *hTERT* gene. We found that X-irradiation did not induce telomere abnormality directly, but significant numbers of telomeres were destabilized in surviving cells, indicating that X-ray-induced altered chromosome organization may affect telomere stability many generations after irradiation.

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MATERIAL AND METHODS

Cell and culture

The immortalized BJ1-hTERT cells used in this study were established by introducing the human telomerase reverse transcriptase subunit (*hTERT*) gene into normal human foreskin fibroblasts.[14] We selected this cell line so that telomere abnormalities might not arise by the shortened telomeres. Cells were grown in α-MEM (Minimum Essential Medium) supplemented with 10% fetal bovine serum, 0.3% glucose, and 50 μg/ml hygromycin B at 37°C in a humidified incubator with 5% CO_2.

X-irradiation

X-rays were delivered from a soft X-ray generator (SOF-

TEX, Japan) operating at 150 kV and 5 mA with a 0.1 mm Cu filter. The cells in the cell culture flasks were placed on an irradiation stage 30 cm from the radiation source. The corresponding dose rate was 0.46 Gy/min.

Cell survival

The frequency of surviving cells was determined by using a colony formation test. In brief, cells in T-25 tissue culture flasks were irradiated with X-ray doses ranging from 1 to 6 Gy. Immediately after irradiation, the medium was removed and cells were washed twice with Ca^{2+}-free phosphate buffered saline (PBS^-). Next, they were immersed in 2 ml of 0.1% trypsin for 1 min. Then, 10 ml of medium was added to the flask, and the cells were collected by pipetting. The viable cells were seeded into 100 mmϕ culture dishes at a density of $2\sim200 \times 10^2$ cells for each dose and incubated for 14 days to form colonies. After the incubation, culture dishes were washed twice with PBS^-, methanol was added, and they kept there for 1 min for fixation. The fixed cells were stained with 5% Giemsa solution, and the colonies consisting of more than 64 cells were counted.

Isolation of survived cell after X-irradiation

Exponentially growing cells were irradiated with 2 Gy of X-rays, and 2×10^3 cells were plated onto 100 mm dishes and incubated for 14 days. All colonies formed were isolated and cultured together as X-ray-surviving cells.

Preparation of metaphase samples of cells

Exponentially growing cells were treated with 0.1 μg/ml colcemid for 1 hour, and mitotic cells were then collected after the flask was shaken to dislodge the metaphase cells. Mitotic cells were subsequently swollen in a hypotonic 0.075 M KCl solution for 20 min at room temperature, and then resuspended in a 3:1 mixture of methanol to acetic acid for 30 min at room temperature. Mitotic cells were dropped onto precleaned glass microscope slides and allowed to dry for 3 days at room temperature.

Analysis of chromosome aberration

Chromosome aberrations 24 hours after X-rays and in X-ray-surviving cells were investigated by Giemsa staining. The glass slides with metaphase chromosomes were dipped in 5% Giemsa solution for 10 min at room temperature. The slides were then washed in distilled H_2O and dried with an air drier, and metaphase chromosomes were viewed under a microscope. We scored chromosome aberrations, including dicentrics, fragments, chromatid gaps, and chromatid breaks. Fragments accompanied by dicentrics were not counted as fragments.

Analysis of telomere abnormality by fluorescence in situ hybridization (FISH)

Analysis of telomere abnormality observed 24 hours after irradiation and in X-ray-surviving cells was performed by centromere- and telomere-FISH. Briefly, the slides were immersed in PBS^- at 37°C for 30 min and dipped in 4% formaldehyde in PBS^- at room temperature for 2 min to fix the chromosome. After the slides were washed 3 times in PBS^- at room temperature for 5 min, they were incubated in 1 mg/ml pepsin solution (PH 2.0) at 37°C for 2 min. They were then rinsed in PBS^- for 10 s and fixed with 4% formaldehyde in PBS^- at room temperature for 2 min. The slides were washed in PBS^- and dehydrated with a series of 1-min washes in 70%, 80%, and 100% ethanol. After a drying with an air drier, 10 μl of the hybridization mixture containing 70% formamide diluted with 1% blocking reagent (Boehringer Mannheim, Gmbh, Germany) in maleic acid buffer, 0.3 μg/ml fluorescein-labeled telomeric peptide nucleic acid (PNA) probe (PerSeptive Biosystems, U.S.A.), and 250 μg/ml TexasRed-labelled centromeric PNA probe (PerSeptive Biosystems, U.S.A.) were added to the slide, which was then covered with a glass cover slip. The slides were then heated at 80°C for 4 min to denature the chromosome and incubated at room temperature for 6 hours. After hybridization, they were washed twice in 70% formamide diluted with 10 mM Tris (pH 7.2) at room temperature for 15 min. After the slides were washed 3 times in a mixture solution containing 0.05 M Tris, 0.15 M NaCl (pH 7.5), and 0.05% tween 20, they were dehydrated again by means of the same ethanol series. After a drying at room temperature, 15 μl of DAPI (500 ng/ml, Vysis, U.S.A.) in Antifade (Molecular Probes, U.S.A.) were added to counterstain the chromosomes, and the slides were covered with glass cover slips. Metaphase chromosomes were viewed under a fluorescent microscope equipped with a dual bandpass DAPI/FITC/RHOD filter set (Olympus, Japan). We scored telomere abnormalities, including chromatid telomere loss, chromatid or chromosomal telomere duplication. Chromosomes, in which one of the chromatids showed no telomere fluorescence, were counted as chromatid telomere loss. Chromosomes with duplicated telomere signals were counted as telomere duplication.

Analysis of telomere intensity by NIH image software

The intensity of each telomere fluorescence in BJ1-hTERT cells was calculated with NIH image software (Wayne Rasband, U.S.A.). Briefly, fluorescence signals were viewed under a fluorescent microscope and fluorescent images were captured by using IP-Lab software (Signal Analytics, U.S.A.). The captured images were converted to black and white images, and the fluorescence intensity of telomere spots was automatically analyzed quantitatively by NIH image software.

RESULT

X-ray sensitivity and isolation of X-ray-surviving cells

Figure 1 shows the dose-response survival data for BJ1-

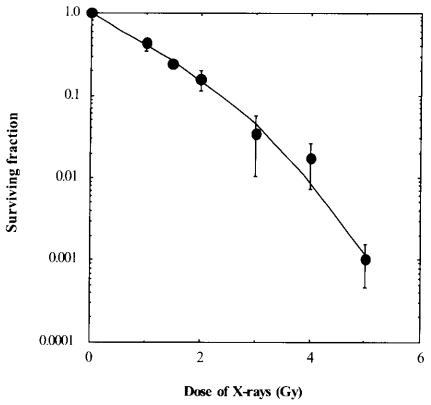

Fig. 1. Surviving fraction of BJ1-hTERT cell irradiated by X-rays. The frequency of surviving cells was determined using a colony formation test.

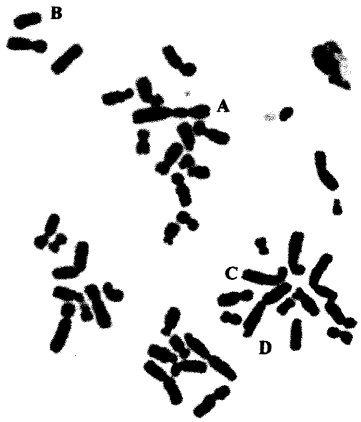

Fig. 2. Giemsa stain analysis of chromosome aberration in BJ1-hTERT cells 24 hours after X-irradiation. A: Dicentric; B: Fragment; C: Chromatid gap; D: Chromatid break

hTERT cells irradiated with X-rays. The 10% survival dose for X-rays was 2 Gy. X-ray-surviving cells were isolated by a collection of the survival colonies 14 days after irradiation with 2 Gy of X-rays. The doubling time of unirradiated BJ1-hTERT cells was approximately 50 hours, and isolated X-ray-surviving cells were estimated to divide 7–9 times after irradiation.

Chromosome aberration

Chromosome aberrations of cells 24 hours after X-ray irradiation were analyzed as shown in Table 1, which reveals that 31.1% of metaphase cells expressed dicentrics, fragments, chromatid gaps, or chromatid breaks. Among the chromosome aberrations observed, dicentrics and fragments were the major aberrations compared to chromatid breaks and gaps (Fig. 2). As shown in Table 1, chromosome aberrations were observed in 13.6% of mitotic cells even at 14 days after X-irradiation. As opposed to chromosome aberration observed 24 hours after X-ray irradiation, delayed chromosome aberrations, which appeared in surviving cells after the irradiation, were mostly chromatid gaps and breaks.

Telomere abnormality

Involvements of telomere abnormality in chromosome aberrations were examined using centromere- and telomere-FISH and results were shown in Table 2. Twenty-four hours after X-ray irradiation, an increased frequency of dicentric chromosomes was detected. All dicentric chromosomes were accompanied with fragments, and no telomere signals were found in dicentrics. These results were in agreement with a previous study.[15] As shown in Table 3, two percent of chromosomes showed telomere loss or telomere duplications 24 hours after irradiation, there were no differences in the fre-

Table 1. Chromosome aberrations after X-irradiation (Giemsa staining).

Dose (Gy)	Time after X-irradiation	No. metaphase counted	No. of abnormal metaphase (%)	Type of aberrations (%)			
				Chromatid-type		Chromosome-type	
				Gap	Break	Dicentric	Fragment[a)]
0		400	7 (1.8)	5 (1.3)	2 (0.5)	0	0
2	24 hours	122	38 (31.1)***	11 (9.0)	5 (4.1)	19 (15.6)	12 (9.8)
2	14 days	206	28 (13.6)**	6 (2.9)	17 (8.3)	0	6 (2.9)

Table 2. Chromosome aberrations after X-irradiation (Centromere & Telomere FISH).

Dose (Gy)	Time after X-irradiation	No. metaphase counted	No. of abnormal metaphase (%)	Type of aberrations (%)		
				Chromosome-type		
				Tricentric	Dicentric	Fragment
0		230	1 (0.4)	0	0	0
2	24 hours	158	36 (22.8)***	1 (0.6)	17 (10.8)	17 (10.8)
2	14 days	233	10 (4.3)**	0	0	3 (1.3)

$^{***}p < 0.01$, $^{**}p < 0.05$, χ^2-test.

Table 3. Telomere abnormality after X-irradiation (Centromere & Telomere FISH).

Dose (Gy)	Time after X-irradiation	No. chromosome counted	No. of chr. with telomere abnormality (%)	Type of aberrations (%)		
				Telomere loss	Telomere duplication	
				Chromatid-type	Chromatid-type	Chromosome-type
0		270	5 (1.9)	4	1 (0.4)	0
2	24 hours	1334	27 (2.0)	18 (1.4)	8 (0.6)	1 (0.08)
2	14 days	429	22 (5.4)**	12 (2.8)	8 (1.9)	2 (0.5)

**$p < 0.05$, χ^2-test.

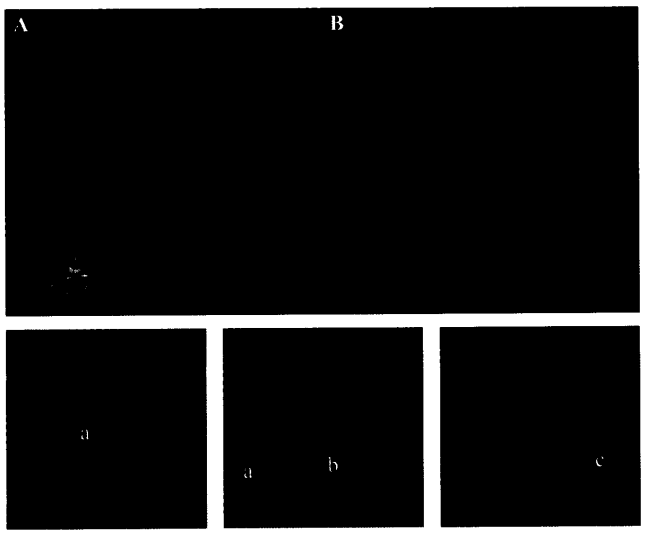

Fig. 3. Centromere- and telomere-FISH analysis of telomere abnormality in BJ1-hTERT cells. A: Unirradiated cells, B: X-ray-surviving cells, (a) Chromatid telomere duplication, (b) Chromosome telomere duplication, (c) Chromatid telomere loss.

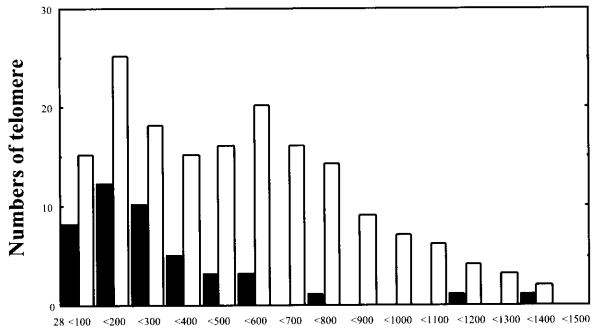

Fig. 4. Distribution of telomere fluorescence intensity in unirradiated BJ1-hTERT cells was compared with that of duplicated telomere in X-ray-surviving cells. Telomere fluorescence images were captured by IP-Lab image software, and fluorescence intensity was analyzed quantitatively by NIH image software. Open bars show the distribution of telomere fluorescence intensity in unirradiated cells. Close bars show the distribution of telomere fluorescence intensity in duplicated telomere.

quency of telomere loss and telomere duplications compared with non-irradiated groups. In contrast, an increased frequency of telomere abnormality was observed significantly in X-ray-surviving cells, and 5.4% of the chromosomes showed that telomere loss or telomere duplications (Table 3 and Fig. 3). Most of the chromosome aberrations with telomere loss were chromatid-type. Table 2 shows that 10.8% of abnormal metaphase showed fragments, but all had telomeres, and therefore they were not counted as chromosomes with telomere loss in Table 3. Distributions of telomere fluorescence intensity in unirradiated BJ1-hTERT cells were presented in Fig. 4. The intensity of each telomere fluorescence was distributed in a range from 31 to 1,400. We compared the distribution of fluorescent intensity of duplicated telomeres, and found that their intensity was relatively lower than that of all telomeres. We also noticed that, in duplicated telomeres, the intensity of proximal telomere, which is proximal to centromere, tended to be lower than the distal telomeres (Fig. 4).

DISCUSSION

In the present study, we analyzed telomere abnormality in chromosomes in normal human fibroblast BJ1-hTERT cells irradiated with X-rays. As we reported previously, normal human diploid cells showed delayed chromosomal instability.[5] However, it is possible that cell division-related telomere shortening by itself affects telomere stability. To avoid that, we have chosen the cell line, in which telomere length is maintained stably. We found that BJ1-hTERT cells were slightly more sensitive to X-rays than normal human diploid cells used previously.

Our results clearly showed that telomere abnormalities were induced in X-ray-surviving cells. Because telomere dysfunction has often been associated with telomere fusions, these observations can explain our previous results, which showed that most of the delayed-induced dicentrics were not accompanied by fragments.[5] It is interesting, as shown in Table 3, that no significant increase in the frequency of telomere abnormality was observed 24 hours after irradiation. In consideration of the results presented in Tables 1 and 2, both cells irradiated at G1 and G2 phases were analyzed 24 hours after irradiation. Although it is also possible that 24 hours after irradiation is not enough to rule out the early effects of radiation, because the doubling time of BJ1-hTERT cells is approximately 50 hours, it is suggested that DNA double-strand breaks by themselves might not induce telomere abnor-

mality directly. Telomere instability in X-ray-surviving cells was manifested as telomere loss and telomere duplication.

DNA double-strand breaks did not induce telomere abnormality directly, so there is two possible mechanisms that might induce delayed telomere instability. The first possibility is that initial damage occurs in subtelomere regions near the telomere site. As our previous studies suggested, DNA double-strand breaks may create nonlethal potentially unstable chromosome regions (PUCR) at the break-rejoining sites through DNA double-strand break repair, which are involved in the initiation and perpetuation of radiation-induced genomic instability.[16,17] Thus, PUCR might be formed in the subtelomere regions and might cause delayed breakage after several cell divisions. As a recent study demonstrated, DNA double-strand break near telomere causes telomere instability.[18] If the broken telomere rejoins to another telomere, the result may be duplicated telomeres. It should be pointed out that most telomere abnormalities observed were chromatid-type; therefore delayed telomere instability could occur during DNA replication or in the G2-phase.

The second possibility is that initial damage might arise in the interstitial chromatin regions, and it might affect the stability of the telomere. Recently, several studies have reported that each chromosome has its own territory.[19] Relative chromosome position is regulated through telomere attachment to the nuclear matrix as well as through the attachment of the interstitial nuclear matrix attachment region (MAR) to the nuclear matrix. If X-irradiation causes a large deletion of interstitial chromatin domain or MAR, it may alter chromatin organization in the nuclei, which in turn could induce telomere reorganization. Alternatively, if an interstitial large deletion causes a change in the chromosome position, it may result in telomere reorganization.

The present findings show, for the first time, that telomeres are destabilized several generations after X-irradiation. Although previous reports have suggested delayed telomere instability by showing a delayed induction of telomere fusions,[13] our results constitute direct evidence that telomeres are destabilized indirectly. End-to-end fusions of shortened or destabilized telomeres are often observed in human tumor cells,[20,21] and these fusions have been thought to perpetuate chromosome instability by inducing the BFB cycle, which may be the mechanism underlying the induction of genomic instability by ionizing radiation.

ACKNOWLEDGEMENTS

This work was supported by a grant for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan and by the Research Project Fund for "Studies on the Molecular Biological Basis for Low-Dose Radiation Effects" from the Japan Atomic Energy Research Institute through a contract with the Nuclear Safety Research Association.

REFERENCES

1. Morgan, W. F., Day, J. P., Kaplan, M. I., McGhee, E. M. and Limoli, C. L. (1996) Genomic instability induced by ionizing radiation. Radiat. Res. **146**: 247–258.
2. Little, J. B. (1998) Radiation-induced genomic instability. Int. J. Radiat. Biol. **74**: 663–671.
3. Chang, W. P. and Little, J. B. (1992) Evidence that DNA double-strand breaks initiate the phenotype of delayed reproductive death in Chinese hamster ovary cells. Radiat. Res. **131**: 53–59.
4. Mothersill, C., Crean, M., Lyons, M., Mcsweeney, J., Mooney, R., Oreilly, J. and Seymour, C. B. (1998) Expression of delayed toxicity and lethal mutations in the progeny of human cells surviving exposure to radiation and other environmental mutagens. Int. J. Radiat. Biol. **74**: 673–680.
5. Roy, K., Kodama, S., Suzuki, K. and Watanabe, M. (1999) Delayed cell death, giant cell formation and chromosome instability induced by X-irradiation in human embryo cells. J. Radiat. Res. **40**: 311–322.
6. de Lange, T. (1998) Telomeres and senescence: ending the debate. Science **279**: 334–335.
7. Kim, S., Kaminker, P. and Campisi, J. (2002) Telomeres, and cancer: In search of a happy ending. Oncogene **21**: 503–511.
8. McClintock, B. (1941) The stability of broken ends of chromosomes in *Zea mays*. Genetics **26**: 234–282.
9. Artandi, S. E., Chang, S., Lee, S. L., Alson, S., Gottlieb, G. J., Chin, L. and DePinho, R. A. (2000) Telomere dysfunction promotes non-reciprocal translocations and epithelial cancers in mice. Nature **10**: 641–645.
10. Samper, E., Goytisolo, E. A., Slijepcevic, P., Buul, P. P. W. V. and Blasco, M. A. (2000) Mammalian Ku86 protein prevents telomeric fusions independently of the length of TTAGGG repeats and G-strand overhang. EMBO Rep. **1**: 244–252.
11. Goytisolo, F. A., Sampler, E., Edmonson, S., Taccioli, G. E. and Blasco, M. A. (2001) The absence of the DNA dependent protein kinase catalytic subunit in mice results in anaphase bridges and in increased telomeric fusion with normal telomere length and G-strand overhang. Mol. Cell Biol. **21**: 3642–3651.
12. Steensel, B. V., Smogorzewska, A. and de Lang, T. (1998) TEF2 protects human telomeres from end-to-end fusions. Cell **92**: 401–413.
13. Day, J. P., Limoli, C. L. and Morgan, W. F. (1998) Recombination involving interstitial telomere repeat-like sequences promotes chromosomal instability in Chinese hamster cells. Carcinogenesis **19**: 259–265.
14. Bodnar, A. G., Ouellette, M., Frolkis, M., Holt, S. E., Chiu, C., Morin, G. B., Harley, C. B., Shay, J. W., Lichtsteiner, S. and Wright,W. E. (1998) Extension of life-span by introduction of telomerase into normal human cells. Science **279**: 349–352.
15. Cornforth, M. N., Meyne, J., Littlefield, L. G., Bailey, S. M. and Moyzis, R.K. (1989) Telomere staining of human chromosomes and the mechanism of radiation-induced dicentric formation. Radiat. Res. **120**: 205–212.
16. Suzuki, K. (1997) Multiple nature of X-ray-induced neoplastic transformation in mammalian cells: Genetic alterations and

instability. J. Radiat. Res. **38**: 55–63.
17. Suzuki, K., Ojima, M., Kodama, S. and Watanabe, M. (2003) Radiation-induced DNA damage and delayed induced genomic instability. Oncogene **22**: 1–6.
18. Lo, A. W., Sprung, C. N., Fouladi, B., Pedram, M., Sabatier, L., Ricoul, M., Reynolds, G. E. and Murnane, J. P. (2002) Chromosome instability as a result of double-strand breaks near telomeres in mouse embryonic stem cells. Mol. Cell Biol. **22**: 4836–4850.
19. Cremer, T. and Cremer, C. (2001) Chromosome territories, nuclear architecture and gene regulation in mammalian cells. Nat. Rev. Genet. **2**: 292–301.
20. Hastie, N. D., Dempster, M., Dunlop, M. G., Thompson, A. M., Green, D. K. and Allshire, R. C. (1990) Telomere reduction in human colorectal carcinoma and with ageing. Nature **30**: 866–868.
21. Counter, C. M., Hirte, H. W., Bacchetti, S. and Harley, C. B. (1994) Telomerase activity in human ovarian carcinoma. Proc. Natl. Acad. Sci. USA **12**: 2900–2904.

Received on October 3, 2003
1st Revision on December 3, 2003
Accepted on December 18, 2003