ISSN (Online) 2287-3406
Journal of Life Science **2024** Vol. 34. No. 6. 365~376 DOI : https://doi.org/10.5352/JLS.2024.34.6.365

Arrest of Cell Growth by Inhibition of Endogenous Reverse Transcription Activity in Cancer and Somatic Cell Lines

Mi-Jeong Kim[1], Sung-Ho Lee[2], Jong-Kuen Park[3] and Byeong-Gyun Jeon[1]*

[1]*Department of Biology Education, Gyeongsang National University, Jinju 52828, Korea*
[2]*Division of Life Science, Gyeongsang National University, Jinju 52828, Korea*
[3]*Department of Chemistry Education, Gyeongsang National University, Jinju 52828, Korea*

Received April 25, 2024 /Revised May 20, 2024 /Accepted May 27, 2024

The present study assessed the cytotoxic effects on cell growth and senescence in human cancer (A-549, AGS, HCT-116, MDA-MB-231, and U 87-MG) and normal (MRC-5 and mesenchymal stem cells) cell lines treated with efavirenz (EFA), an inhibitor of non-nucleoside reverse transcriptase (RTase). Following EFA treatment, the half-maximal inhibitory concentration (IC_{50}) values were approximately 15 μM, and the IC_{50} value was significantly ($p<0.05$) lower in the cancer cell lines, compared to normal cell lines. After determining the IC_{50} values against EFA, each cell line was treated with 15 μM EFA for up to one week. Significant ($p<0.05$) decreases in endogenous RTase and telomerase activity were observed in the cancer cell lines. RTase and telomerase activity were absent or detected at very low levels in both EFA-untreated and treated MRC-5 and MSC normal cells. The cell doubling time (CDT) was also significantly ($p<0.05$) prolonged by the decreased cell growth rate in the EFA-treated cancer cell lines compared to the untreated cell lines. Furthermore, EFA-treated cancer cells displayed a high number of cells with a high intensity of senescence-associated ß-galactosidase activity (SA-ß-gal activity), compared to the untreated cells. The present study showed that inhibition of RTase activity induces cellular senescence and arrests cell growth in human cancer cell lines; however, normal cell lines showed greater tolerance against EFA. RTase treatment could offer optional chemotherapy for cancer treatment in human cancer cell lines with high RTase activity.

Key words : Cancer cells, cell growth, human, reverse transcriptase

Introduction

The basic cellular properties of cancer cells are characterized by the unlimited cell growth and metastasis capacity acquired with mainly various epigenetic alterations, such as DNA methylations or histone modifications, and (or) genetic alterations [16]. Recently, it has been re-highlighted that genetic diversity and modification are highly contributed by transposable elements (TEs) in the whole human genome [1, 12]. The TEs in the human genome consist of mainly Class I (retrotransposons) and partially Class II (DNA transposons). The part of Class I retrotransposons in the eukaryotic cell is transcribed to RNA intermediate, subsequently re-converted into a complementary strand of DNA, and these cDNAs can be transposed into other genomic locations with a “copy and paste” mechanism [11]. The DNA transposition of the retrotransposons can be incorporated into the regulatory regions, such as promoter and enhancer, or exon region of their DNA, and mutations of genes by DNA editing in the genome can be induced by retrotransposon mechanisms [26]. Further, the retrotransposons, previously called as such as ‘junk DNA’ and ‘molecular parasites’ can be also changed to structural modifications of DNA, such as DNA methylation and histone modification in eukaryotic cells [9]. Thus, it has been continually demonstrated that gene transcription and expression may be dramatically changed by the transposition of the retrotransposons [12, 26]. Numerous studies have also highlighted that the transposition of retrotransposons is remarkably associated with various diseases in humans, including cancer [21].

After transcription of the retrotransposons in the eukaryotic cells, the converting retrotransposons into cDNA is commonly catalyzed by the endogenous non-telomeric reverse transcriptase (RTase) activity that is usually encoded by Class

***Corresponding author**
Tel : +82-55-772-2236, Fax : +82-55-772-2239
E-mail : bgjeon@gnu.ac.kr

I retrotransposons itself, and the endogenous RTase activity is positively necessary for the transposition of retrotransposons into other genomic locations with a cell [29]. Further, the endogenous RTase activity is commonly expressed in RNA viruses, including retroviruses and coronaviruses for DNA intermediates and cDNA. Thus, the several inhibitors of RTase activity that can limit the production of DNA intermediates from retrotransposons and are widely used for the treatment of acquired immune deficiency syndrome (AIDS) by infection of human immunodeficiency virus (HIV) retrovirus [13]. The nucleoside RTase inhibitors, such as abacavir (Ziagen®) and lamivudine (Epivir®), and non-nucleoside RTase inhibitors, such as efavirenz (EFA, Sustiva®) and nevirapine (NVP, Viramune®) are commonly used for blocking of RTase activity [33]. Compared with nucleoside RTase inhibitors that competitively bind at the active site of the RTase, the non-nucleoside RTase inhibitors non-competitively bind at a hydrophobic pocket site distant from the catalytic active site of the RTase enzyme and disrupt the activity of RTase as per the altering active site conformation [30].

In eukaryotic cells, a high level of endogenous RTase activity is consistently expressed in especially immortal cells, such as including embryos, stem cells, and cancer cells [4, 18, 36]. And, previous studies have shown that stemness characterizations are gradually decreased as per the malfunction of retrotransposons by inhibition of RTase activity with NVP in the human mesenchymal stem cells [22]. Further, inhibition of RTase activity was induced in the arrest of cell growth and cellular differentiation in cancer cells with a high level of RTase activity [28]. Thus, it has been suggested that the inhibitors of RTase activity have been considered as a potential agent for cancer treatment and chemotherapy. The retrotransposons in the eukaryotic cells possess high functions on the basic cellular properties, however, the detailed molecular mechanism(s) and function(s) of retrotransposons are fully elucidated and presently under debate. Most importantly, the investigation concerning the cellular cytotoxicity and growth in the normal somatic cells was not fully examined in the cancer therapy and treatment with RTase inhibitors.

To further investigate the roles of retrotransposons and RTase activity on the basic cell growth and characterizations in the various tissues-derived cancer and normal somatic cell lines, the cells were exposed to EFA, a kind of non-nucleoside RTase inhibitor. The half-maximal inhibitory concentration (IC_{50}) value was first determined by cell viability assays in cancer and normal cell lines. The RTase activity was detected by PCR-based protocol in the normal cell lines treated with EFA, and the basic cancerous cell properties, including cell growth rate by cell doubling time (CDT) assay, relative telomerase activity by real-time quantitative telomeric repeat amplification protocol (RQ-TRAP) assay and the cellular senescence assay using expression of galactosidase. Based on the present results comparing cell growth in human cancer and normal cells treated with EFA, we have examined another possibility in cancer treatment using RTase inhibitors.

Materials and Methods

Cultivation of cancer and normal cells

The human cancer cell lines used in the present experiment were A549 lung adenocarcinoma, AGS gastric adenocarcinoma, HCT-116 colorectal carcinoma, MDA-MB-231 breast adenocarcinoma, and U 87-MG brain glioblastoma derived from the ATCC (USA). The human normal cell lines were MRC-5 fetal lung fibroblasts (ATCC, USA) and mesenchymal stem cells (MSC) isolated from dental tissue of the third molar. The cellular properties of the isolated MSC were examined in the early studies [17, 18]. The basic cell culture media was advanced-Dulbecco's modified eagle medium (A-DMEM, Gibco, USA) supplemented with 3% fetal bovine serum (FBS, Gibco, USA), 1% L-glutamine and 1% penicillin-streptomycin. All cancer and normal cells were grown at 37℃ in a humidified atmosphere of 5% CO_2 in an incubator. The cells at 70−80% confluence were dissociated with 0.25% trypsin-EDTA solution (Gibco, USA), and harvested at 300 ×g for 5 min. And the cells were sub-cultured for further experiment. The EFA stock solution was prepared by dissolving in dimethyl sulfoxide (DMSO, Sigma, USA) at 100 mM and freshly added to the cell culture medium.

Analysis of IC_{50} value

The cellular cytotoxicity against EFA was examined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma, USA) assay and the IC_{50} value in each cell lines was determined as per MTT assay. In brief, each of the cells was transplanted into a 6-well cell culture plate at the density of 1×10^4 cells per well, cultured in A-DMEM containing 0 (untreated control), 3.125, 6.25, 12.5, 25, and 50 μM EFA for 7 days. Following treatment, the wells were washed with Dulbecco's phosphate buffered saline (D-PBS) three times, and incubated with 1 ml of 5 mg/ml MTT stock solution at 37℃ for 4 hr After washed with D-PBS, the formazan in each well was extracted with 200 μl

DMSO solution for 15–30 min. The melted formazan solution was transferred into a new 96-well plate, and the quantity of soluble formazan was measured with a plate reading spectrophotometer (Microdigital, Korea) at a wavelength of 570 nm. The cell viability rate in the untreated treatment was calculated as 100% for relative comparison with each treatment at least 3 replications. Moreover, the morphological changes of the cells in each treatment were examined with an inverted microscope (Nikon, Japan) placed with a CCD camera and image program (IMT, Korea).

Analysis of RTase activity

The endogenous RTase activity in the cells treated with EFA was measured by PCR-based protocol as previously described by Jeon et al. (2011) with minor modifications. Briefly, the total protein of each treatment was 1× CHAPS cell lysis buffer (TRAPeze®, Cell Signal Tech., USA) containing 100–200 U/ml RNase inhibitor (Gibco, USA). Firstly, the samples were incubated at 4℃ for 30 min, and subsequently centrifuged at 12,000 ×g for 20 min at 4℃, and approximately 70% of the supernatant of the cell lysis was transferred into a new tube. The concentration of total protein was calculated with a spectrophotometer (Microdigital, Korea, USA). The cDNA synthesis was followed with cell extract with RTase activity. The reactions for cDNA synthesis were contained with 2 μl of reverse transcription buffer (Qiagen, USA), 10 μg of total protein derived from each sample, 10 ng of bacteriophage MS2 RNA (Sigma, USA), 1 mM of dNTP mixture (Gibco, USA), 2 U of RNase inhibitor (Gibco, USA), and 30 pmol of MS2 reverse primer in a final volume of 20 μl, and were incubated at 37˚C for 1 hr. The cDNA samples were amplified with a PCR pre-mix kit (Intron, Korea) containing 10 pM each of forward (5′-CCTCCTCTCT GGCTACCGA-3′) and reverse (5′-ACAGGCAGCCCGATC TATTT-3′) MS2 primers. The PCR protocol consisted of denaturation for 30 sec at 95℃, annealing of 30 sec at 58℃, and extension of 45 sec at 72℃ in 35 cycles. The PCR product and fragment were designed with 296 bp located at 2225–2521 base from the 5’ end of the MS2 RNA checked on the 1.0% agarose gel. The quantification of the PCR product was measured by a second derivative method using quantification software equipped with LightCycler real-time PCR machine (Rotor-Gene Q, Qiagen, USA).

Analysis of cell growth rate

After the determination of the IC_{50} value in each cell line, the cell lines were treated at 15 μM EFA for 7 days. The effect on the growth inhibition by EFA treatment was analyzed by the evaluation of the cell doubling time (CDT) assay. Shortly, the 1×10^4 cells per well were transferred into a 6-well plate, and cultured in the A-DMEM media containing 15 μM EFA for 7 days. The cell culture media was changed twice a week. After 7 days, the cells were trypsinized and harvested, and the number of cells was measured with a hemocytometer. The CDT was calculated following the formula: CDT = ln (log N_t/N_o)/t, where t is the cell culture time between N_o and Nt, and N_o and N_t are the initial and final cell numbers.

Analysis of telomerase activity

The analysis of relative telomerase activity in the EFA-treated cells was employed with a relative-quantitative telomerase repeat amplification protocol (RQ-TRAP) assay, applying real-time PCR protocol and machine (Rotor-Gene Q, Qiagen, USA). Shortly, the sample cells were collected and lysed with CHAPS cell lysis buffer for extraction of total protein from each treatment, as mentioned above. Following the measure of protein concentration with a spectrophotometer (Microdigital, Korea), an RQ-TRAP assay with a real-time PCR machine (Rotor-Gene Q, Qiagen, USA) was carried out in each treatment. Each reaction contained 1 μg total protein, 0.02 μg of telomerase TS (5′-AATCCGTCGGAGCA GAGTT-3′) primer, and 0.04 μg of anchored return ACX (5′-GCGCGGCTTACCCTTACCCTTACCCTAACC-5′) primer in the 20 μl Rotor-Gene™ SYBR green mixture (Qiagen, USA). The amplification protocol of telomerase consisted of 94℃ for 30 sec and 60℃ for 90 sec in 40 cycles. The relative level of telomerase activity was measured by a value of cycle threshold with Rotor-Gene Q software (Qiagen, USA), referring to the level of telomerase activity in the MRC-5 fetal fibroblasts.

Analysis of cellular senescence

After treatment of EFV for 1 week, the cellular senescence (aging) was examined with the senescence-associated β-galactosidase (SA-β-gal) activity using a SA-β-gal staining kit (Cell Signaling Technology, USA), as per the manufacturer’s protocol. In Brief, the untreated control and EFA-treated cells were washed with D-PBS twice, and fixed with fixative solution for 30 min. After being washed with D-PBS, each cell was stained with the SA-β-gal staining solution at 37℃ overnight. After being again washed with D-PBS, each cell was observed under an inverted microscope (Nikon, Japan) placed with a CCD camera and image program (IMT, Korea).

The cells presented with blue color were considered as a senescent cell.

Statistical Analysis

All treatment group was independently performed over at least three replicates, and all data are exhibited as mean ± standard error of the mean (mean ± SEM). The statistical significance differences were carried out by one-way analysis of variance (ANOVA) applying SPSS statistics software (version 15.0, IBM, USA). The level of statistical significance was examined at $p<0.05$.

Results

Determination of IC_{50} value

Following EFA administration for 7 days, the cell growth pattern was examined under an inverted microscope, as representatively displayed in Fig. 1. And the cell viability rate was investigated with MTT assay in each cell line, as shown in Fig. 2A. The cell viability rate was gradually decreased along with increasing EFA treatment concentration. In particular, the viability rate in the cancer cell lines was highly down-regulated at over 25 μM EFA (Fig. 2A). Further, the IC_{50} value was determined in each cell line, based on the survival rate by MTT assay. The IC_{50} value was 12.8±1.33, 10.9±1.02, 15.3±1.44, 13.9±1.91 and 14.7±2.23 μM in AGS, A-549, MDA-MB-231, HCT-116, and U 87-MG cancer cell lines, respectively (Fig. 2B). Whereas, the IC_{50} value was 22.5±1.23 and 27.4±1.21 in the MRC-5 fibroblasts and MSC cell lines, respectively. The IC_{50} value against EFA was significantly ($p<0.05$) exhibited at a high value in the normal cell lines, including MRC-5 fibroblasts and MSC, compared with those of cancer cell lines.

Analysis of RTase activity

To analyze RTase activity, a PCR-based protocol using MS2 RNA was carried out in each cell line treated with EFA-treated cell lines. The RTase activity in the positive control using commercial RTase enzyme was considered as 100% for relative comparison with each cell line, as shown in Fig 3A and B. The RTase activity was 81.7±5.23, 73.5±3.20, 82.1±3.13, 67.9±2.99, and 57.5±1.23 in the untreated

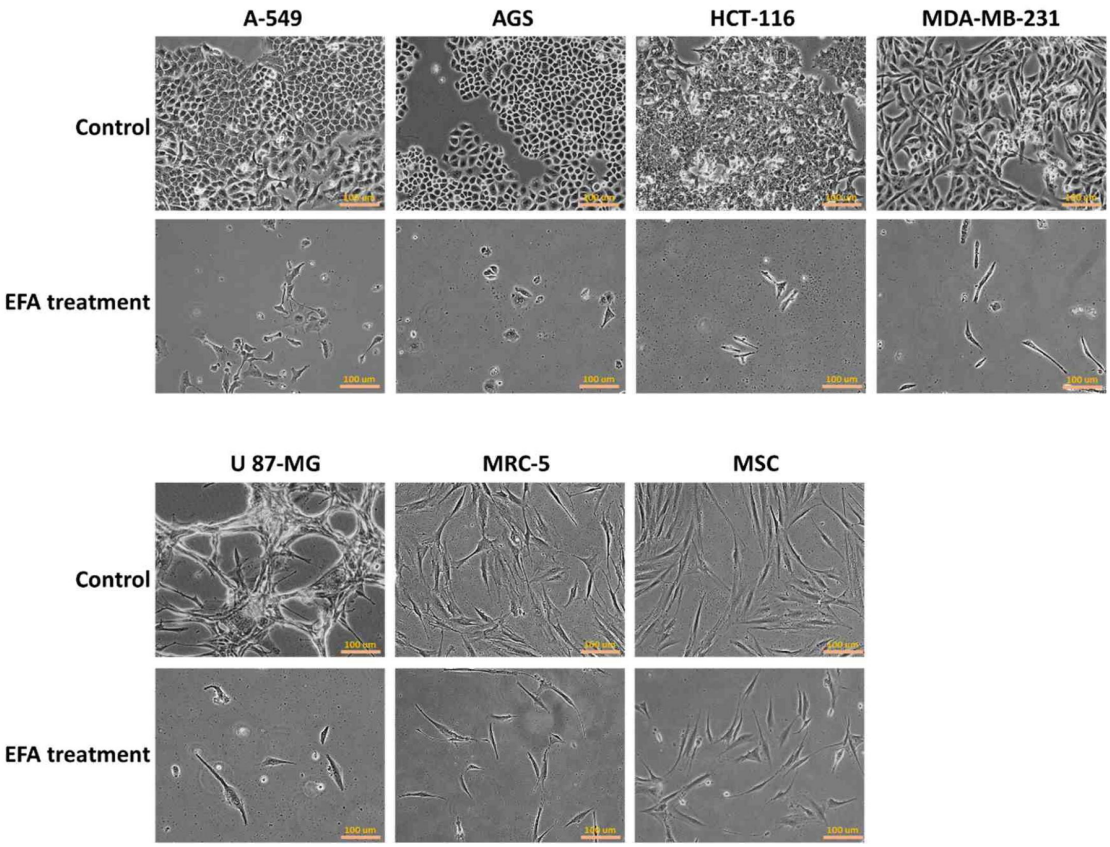

Fig. 1. Pattern of cell growth in AGS, A-549, MDA-MB-231, HCT-116, U 87-MG, MRC-5, and MSC cell lines treated with 25 μM EFA for 7 days (×200). Scale bars; 100 μm.

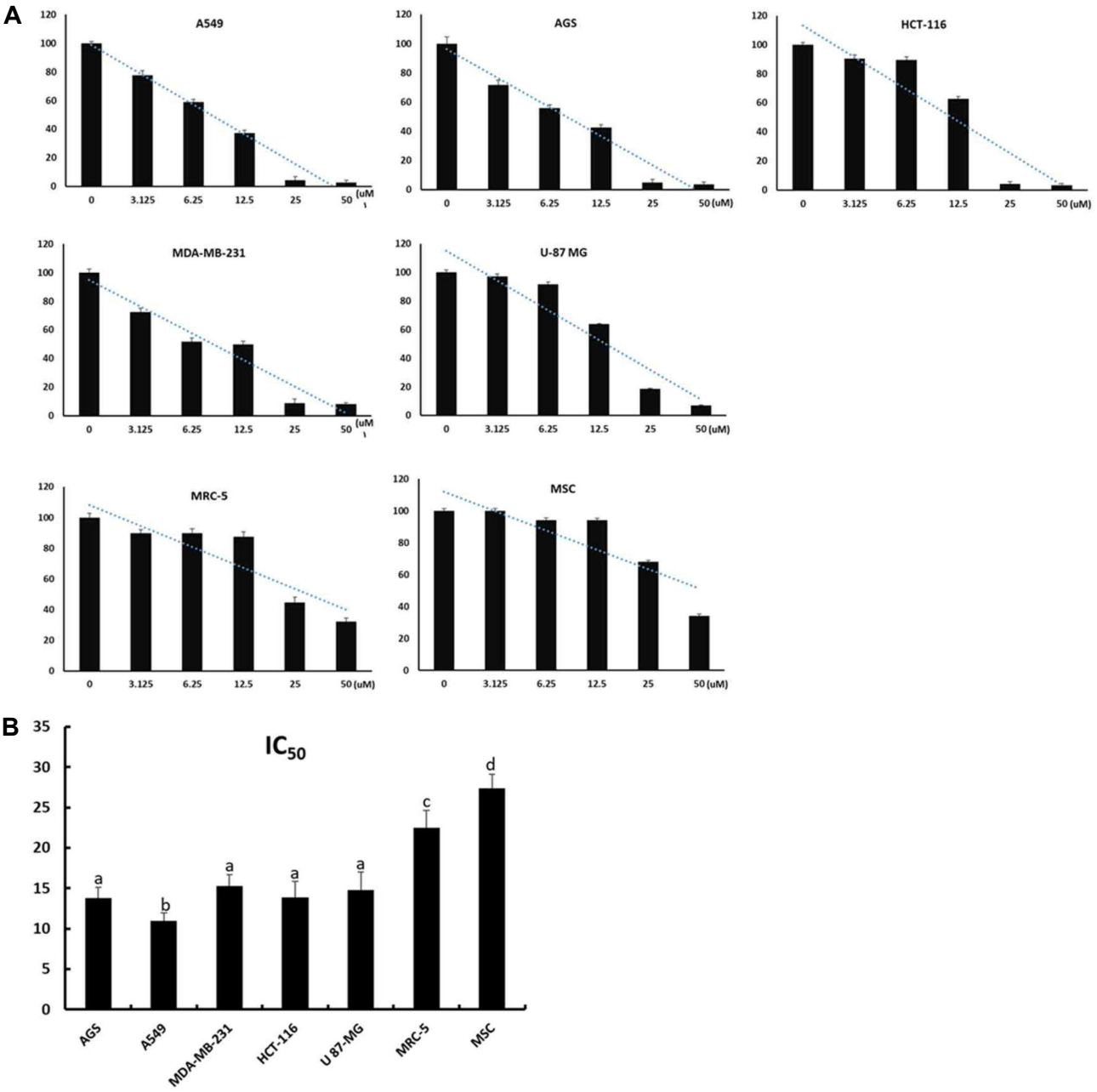

Fig. 2. Analysis of cell growth and determination of IC_{50} values by MTT assay in AGS, A-549, MDA-MB-231, HCT-116, U 87-MG, MRC-5, and MSC cell lines treated with EFA. (A) Cell growth curves by MTT assay in each cell line. (B) Mean ± SEM of IC_{50} values of each cell line in triplicate. a, b, c, and d indicate significant (p<0.05) differences among each cell line.

A-549, AGS, HCT-116, MDA-MB-231 and U 87-MG cell lines, respectively. The RTase activity was not detected in the normal cell lines, including MRC-5 and MSC cell lines. Whereas, the RTase activity was 9.0±1.18, 3.2±1.25, 3.1± 2.16, 0, and 0 in EFA-treated A-549, AGS, HCT-116, MDA-MB-231, and U 87-MG cell lines, respectively. The RTase activity was significantly (p<0.05) decreased by the EFA treatment in the cancer cell lines, compared with their untreated counterparts.

Analysis of cell doubling time

Following the determination of IC_{50} value in each cell line, the cells were treated at 15 μM EFA for 7 days. The effect on the cell growth by EFA treatment was analyzed by cell doubling time (CDT) assay with counting cell number, as shown in Fig. 4. The mean CDT was 25.9±2.98, 30.1±3.45,

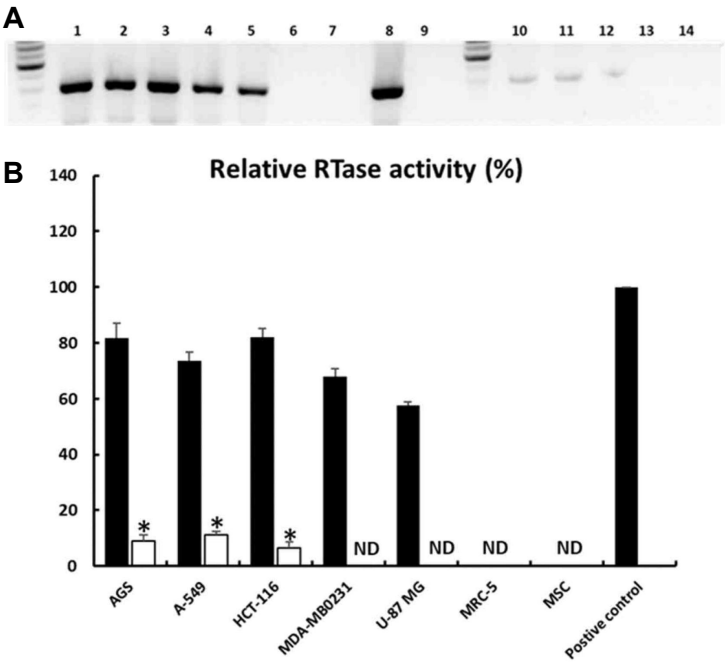

Fig. 3. Analysis of PCR-based RTase activity using MS2 RNA and their primers. (A) Representative gel image of RTase activity with each cell extract of untreated (1−7 lane) and EFA-treated (10−14 lane) cell line. Lane 1−7: untreated A-549, AGS, HCT-116, MDA-MB-231, U 87-MG, MRC-5, and MSC, lane 8; negative control with H_2O instead of cell extract, lane 9; positive control using 1 IU of commercial RTase enzyme, Lane 10−14; EFA-treated A-549, AGS, HCT-116, MDA-MB-231, and U-87-MG. (B) Mean±SEM (n=3) of relative RTase activity in the untreated (black bars) and EFA-treated (white bars) cell lines. The RTase activity in positive control was considered as 100% for relative comparison with each cell line. *, Significant (p<0.05) difference between the untreated and EFA-treated treated cell line. ND: Not detected.

25.9±3.38, 43.0±1.29 and 50.6±3.67 hr in the untreated control A549, AGS, HCT-116, MDA-MB-231 and U 87-MG cancer cell lines, respectively. The mean CDT was 44.7±2.28 and 38.3±2.67 hr in untreated MRC-5 and MSC cell lines, respectively. Whereas, the mean CDT was 62.4±2.87, 64.9±1.26, 59.8±3.76, 62.2±3.89 and 61.2±2.54 hr in the EFA-treated A549, AGS, HCT-116, MDA-MB-231 and U 87-MG cancer cell lines, and the mean CDT was 51.0±2.01 and 48.6±2.02 hr in EFA-treated MRC-5 and MSC cell lines, respectively. The mean CDT was significantly (p<0.05) extended by the decrease of cell growth in the cell lines treated with EFA.

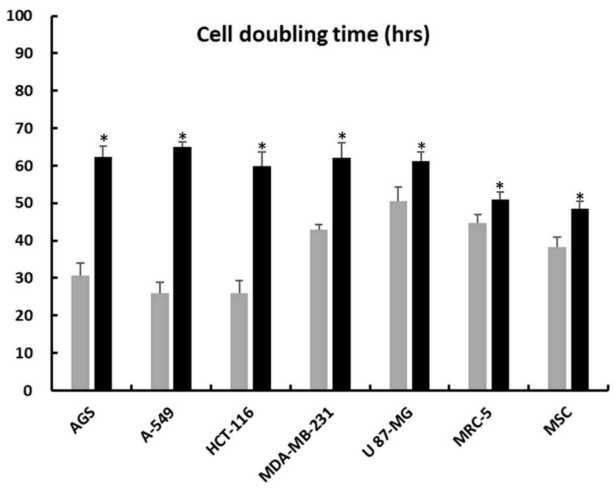

Fig. 4. Analysis of cell doubling time (CDT) in untreated (grey bras) and 15 μM EFA-treated (black bars) AGS, A-549, HCT-116, MDA-MB-231, U 87-MG, MRC-5, and MSC cell lines. Asterisks (*) indicate a significant (p<0.05) difference between untreated control and EFA treatment.

Analysis of telomerase activity

Following 15 μM EFA treatment, the level of telomerase

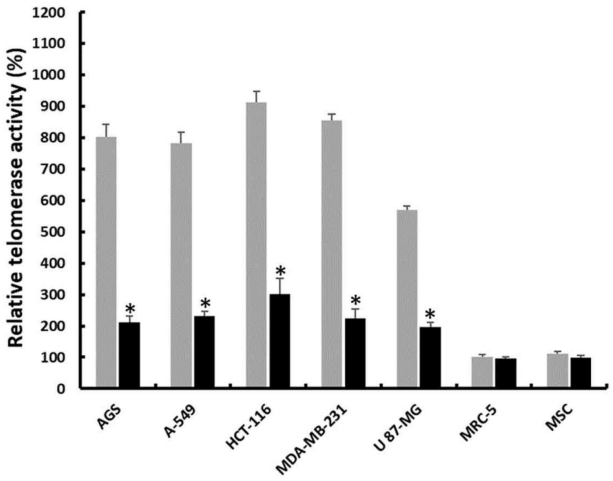

Fig. 5. Analysis of telomerase activity by RQ-TRAP in the untreated control (grey bars) and 15 μM EFA-treated (black bars) AGS, A-549, HCT-116, MDA-MB-231, U 87-MG, MRC-5, and MSC cell lines. The telomerase activity in untreated MRC-5 was measured as 100% for relative comparison with each cell line. Asterisks (*) indicate a significant (p<0.05) difference between untreated control and EFA treatment.

activity for a useful characterization of malignancies was measured by RQ-TRAP assay, as shown in Fig. 5. The relative telomerase activity was 801±41.4, 782±35.9, 913±33.3, 855±19.2, 568±13.6, 100±8.8 and 112±6.7 % in the untreated AGS, A-549, HCT-116, MDA-MB-231, U 87-MG, MRC-5 and MSC cell lines, respectively. The level of telomerase activity in the normal cell lines, including MRC-5 fibroblasts and MSC was detected at a basal level. However, the level of telomerase activity in the cancer cell lines, including A-549, AGS, and HCT-116 was detected at a high level, compared with those of normal cell lines. And the relative telomerase activity was 210±20.8, 232±25.2, 301±50.7, 223±30.8, 197±13.5, 95±7.3 and 99±7.6 % in the EFA-treated AGS, A-549, HCT-116, MDA-MB-231, U 87-MG, MRC-5 and MSC cell lines, respectively. The telomerase activity was significantly (p<0.05) down-regulated in the EFA-treated cancer cell lines, and the level of telomerase activity tended to be slightly decreased in the EFA-treated MRC-5 fibroblasts and MSC, than those of untreated MRC-5 fibroblasts and MSC, but was not significantly (p<0.05) different.

Analysis of SA-β-GAL activity

The senescence-associated β-galactosidase activity (SA-β-GAL activity) highly expressed in senescent or apoptotic cells was detected in the EFA-treated cell lines, as shown in Fig. 6. The high intensity and frequency of cells stained with blue color was generally considered to be a cell with high SA-β-GAL activity, and the EFA treatment was induced into the increase of cell with high SA-β-GAL activity in cancer and normal cell lines. The frequency of cells with high SA-β-GAL activity was occasionally observed in the untreated normal MRC-5 fibroblasts and MSC, implying that the cells already reached the senescent status before EFA treatment. Further, the cell shape was gradually changed to enlarged, flattened, and star-shaped morphological alternation in the EFA-treated cells, compared to those of untreated control cells.

Discussion

The present study investigated the growth inhibition effect in the various types of cancer (A-549 lung adenocarcinoma,

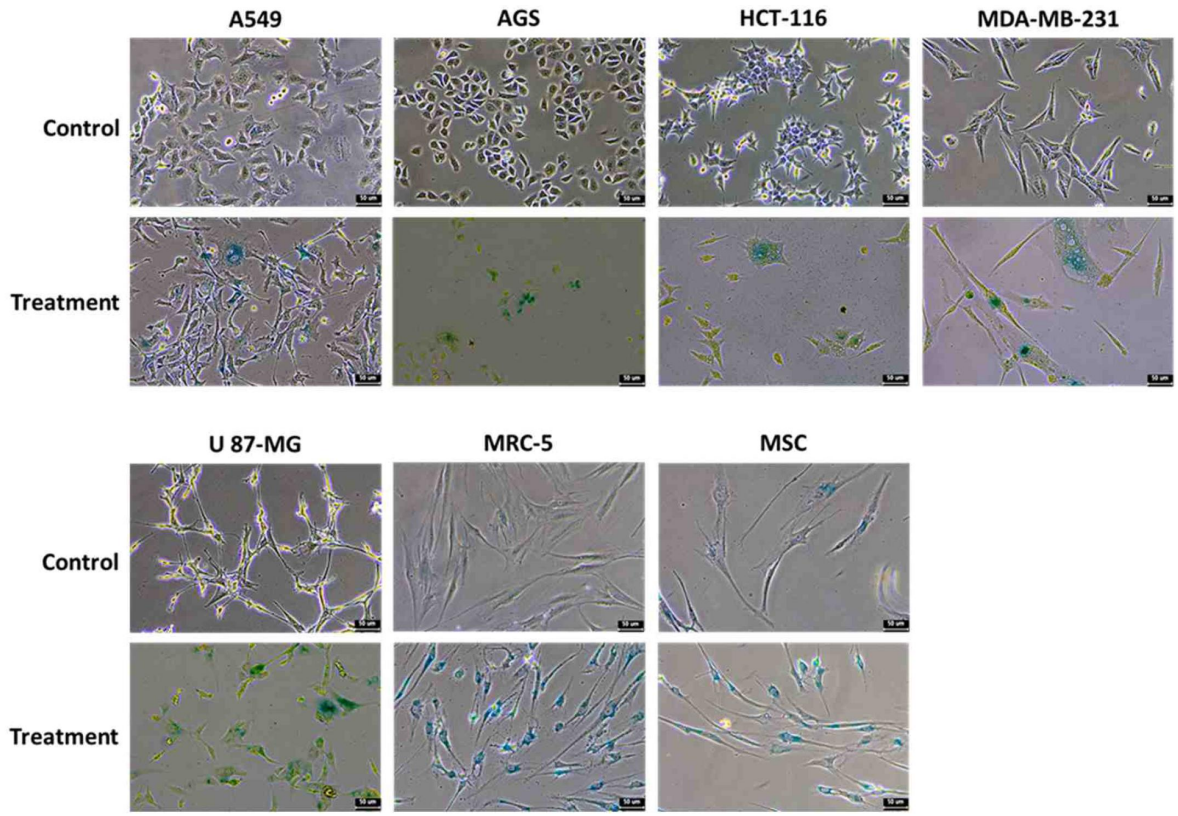

Fig. 6. Changes of cell morphology and SA-β-GAL activity in the untreated control and 15 μM EFA-treated AGS, A-549, HCT-116, MDA-MB-231, U 87-MG, MRC-5, and MSC cell lines (×200). The high incidences of SA-β-GAL activity (blue color) were expressed in the cell lines treated with 15 μM EFA. Scale bars; 50 μm.

AGS gastric adenocarcinoma, HCT-116 colon carcinoma, MDA-MB-231 breast adenocarcinoma, and U 87-MG brain glioma) and normal somatic cell lines (MRC-5 lung fibroblasts and mesenchymal stem cells, MSC) treated with EFA, a kind of non-nucleoside RTase inhibitor commonly used for antiretroviral medication, such as human immunodeficiency virus (HIV). Following EFA treatment for 1 week in cancer and normal cell lines, the basic cellular properties, including cell growth rate, level of telomerase activity, and frequency of cellular senescence. When the IC_{50} value was estimated by MTT assay in cancer and normal cell lines, the lower IC_{50} value was exhibited in the various types of cancer cell lines than those of normal cell lines (MRC-5 fibroblasts and MSC). Further, the present study demonstrated that inhibition of RTase activity using EFA is highly induced in the decreased rate of cell growth, down-regulation of telomerase activity, and high incidence of cellular aging or senescence in the cancer cell lines. While, the delay of cell growth and cellular senescence is more weakly exhibited in the normal cell lines, including MRC-5 fibroblasts and MSC.

A retrovirus, including HIV, belongs to the viral family Retroviridae and is a type of reverse-transcribing virus with a single-stranded RNA genome. When the virus invades a host cell, the viral RNA genome is converted into double-stranded DNA via RNA-DNA hybrid by their endogenous reverse transcriptase (RNA-dependent DNA polymerases, RTase) activity. Their gene replication and proliferation rely essentially on the RTase activity in the virus [8]. Thus, RTase inhibitors of several types, including nucleoside analog RTase inhibitors, nucleotide analog RTase inhibitors, non-nucleoside RTase inhibitors, and nucleoside RTase translocation inhibitors are commonly applied for the treatment of HIV. Azidothymidine (AZT, Zidovudine®) was first approved for HIV treatment and AIDS, as a kind of nucleoside analog RTase inhibitor [13]. The EFA and NVP, known as a representative non-nucleoside RTase inhibitor that allosterically binds to a distinct site away from the binding pocket at the active site of the RTase enzyme inhibit the RTase activity and also used for HIV and AIDS treatment [30].

The human genome is commonly composed of protein-coding and noncoding DNA sequences, and the major components of the noncoding DNA sequences (approximately over 45%) are transposable elements (TEs), including retrotransposons (class I TEs) and DNA transposons (class II TEs), previously known as junk DNA [1, 12]. Further, the major part of TEs are DNA retrotransposons and are usually transcribed into single-stranded RNA intermediates. Further, the single-stranded RNA intermediates are converted to double-stranded complementary DNA (cDNA) by endogenous RTase activity in the cell itself and incorporated into a new DNA sequence, known as the “copy-and-paste” mechanism in eukaryotic cells, including humans. Whereas, class II transposons are incorporated with a "cut and paste" mechanism [11]. Thus, the retrotransposon DNA can lead to the expansion of their genome, unlike transposons. Recently, numerous studies have highlighted that retrotransposon is highly related to the modification of gene expression by insertion of the retrotransposon cDNAs into promoters, exons, and other regulatory parts [26]. Further, it has been shown that endogenous RTase activity is essential for the synthesis of double-stranded cDNA from transcribed single-stranded retrotransposons RNAs [25]. The intrinsic role and function of endogenous RTase activity in the cell are still unclear until now, however, a high level of RTase activity is expressed in the embryonic cells, germ cells, and cancer cells with carcinoma cells with especially unlimited cell growth capacity [4, 17, 36]. Further, it has been previously reported that the inhibition of endogenous RTase activity with NVP and AZT RTase inhibitors is induced to inhibit cell growth in the human germ cells and cancer cells, like inhibition of viral growth [6, 28]. And the early another study has shown that retardation of cell growth and loss of cellular characterizations of mesenchymal stem cells by inhibition of endogenous RTase activity with NVP is induced in the adult mesenchymal stem cells derived from human wisdom teeth [22]. The present study has also shown that the inhibition of cell growth is displayed in the cancer and normal cell lines treated with EFA. Until now, the number of reports and our results have shown that inhibition of RTase activity is induced into delayed or arrested cell growth. Even though the explanation concerning the causes of the delayed cell growth by RTase inhibition is not fully demonstrated in eukaryotic cells, previous reports have suggested that inhibition of the endogenous RTase activity in cancer cells may result in cell growth and differentiation by epigenetic modification with retrotransposons [27]. The inhibition of high RTase activity in tumor cells also leads to the arrest of cell growth and cellular differentiation by damage to the formation of double-stranded RNAs and the subsequent production of small regulatory RNAs, such as microRNA [27]. Further, it has been shown that inhibition of RTase activity with dapivirine is induced in the arrest of cell growth by the activation of JNK and PI3K/Akt pathway [24]. Whereas, up-regulation of RTase expression was induced in tumor growth and metastasis by the

production of the reactive oxygen species (ROS), and induction of epithelial-mesenchymal transition (EMT) [2]. Based on studies until now, we estimated that the retrotransposons contribute to the various roles and functions, including expansion of the gene families and expression regulation of various genes. Thus, the inhibition of retrotransposons and RTase activity may result in diverse cell properties as well as cell growth capacity related to gene expression.

Further, the present results have shown that inhibition of RTase activity by EFA treatment induced cellular senescence and aging via SA-β-GAL assay, as well as anti-proliferative potential. The β-galactosidase in the cell is an enzyme for the cleavage of disaccharide lactose to galactose and glucose. In the eukaryotic cell, the β-galactosidase enzyme is highly exhibited in the aged and senescent cells, and the assay of β-galactosidase expression and activity is a great protocol for the investigation of cellular senescence and aging [19, 22]. The others reports have also shown that the treatment of abacavir as an RTase inhibitor is induced to the cellular senescence in the prostate cancer cells [6]. Generally, cellular senescence is the loss of cell growth capacity and arrest of the cell cycle, and the apoptosis stage is the programmed cell death as per sequential cellular events from the senescent stage [7]. Further, it has been reported that the senescent cells are typically changed to enlarged, flattened, and vacuolated morphological alternations [19]. The present study has shown that enlarged and star-shaped cells are observed in the EFA-treated cells, implying that the cells are reached to the senescence. Thus, even though the present study did not investigate the apoptotic signal pathway and process, the cells at the senescent stage by EFA treatments should have reached to apoptosis stage. Taken together, inhibition of RTase activity by EFA or other RTase inhibitors can induce cellular senescent and apoptotic stages as well as delayed cell growth.

In the human, previous studies have reported that RTase activity is mainly expressed in the especially immortal cells, such as tumor and stem cells than those of normal somatic cells with limited cell growth capacity [22, 35]. The present study also shows that RTase activity is more highly expressed in the various cancer cell lines than those of normal cell lines, such as MRC-5 fibroblasts and MSC. The early study has also shown that RTase activity is detected at a very low or undetected in normal cell lines [22]. However, the cell growth rate in the normal MRC-5 fibroblasts and MSC without expression of RTase activity was interestingly arrested by inhibition of RTase activity with EFA treatment. The normal somatic cell lines, such as MRC-5 with a very low RTase activity are still doubtful that their cell growth is arrested by RTase inhibitors. However, the present study demonstrated that the cellular cytotoxicity against EFA is slightly higher in the cancer cell lines than those of normal cell lines. Another report has shown that apoptotic cell death is also higher in pancreatic cancer cells than in normal fibroblasts [14]. The reason or analysis of the different cytotoxic effects against EFA is still unclear and the induction of apoptosis as well as arrest of cell growth by inhibition of RTase activity is not fully demonstrated in the present study. The basic and intrinsic mechanism(s) concerning the roles and functions of RTase and retrotransposons on cell growth will further be examined in normal somatic cells as well as cancer cells.

In eukaryotic cells with linear DNA, the end of chromosomes consists of repeats of non-coding DNA sequences, called telomeres and the telomere repeats with numerous proteins generally protect against the loss and degradation of DNA with each gene [31]. However, the telomeric repeats are gradually shortened by cell division with semi-conservative DNA replication at the 3'-end of the chromosomes, known as replicative senescence, and the cells with shortened telomere end limit capacity of cell division, eventually enter the cellular senescence and apoptosis in the normal somatic cells [20]. Further, the immortal cells, including cancer and undifferentiated embryonic stem cells possess a high level of telomerase activity that their telomeric repeats can be continually lengthened or maintained with telomerase reverse transcriptase (TERT) activity. Thus, the telomerase reverse transcriptase (namely, telemetric RTase) is an RNA-dependent DNA polymerase using telomerase RNA (TERC), a kind of reverse transcriptase [10]. In the present study, the telemetric RTase (telomerase) activity was highly expressed in the cancer cell lines, compared with those of normal cell lines. Besides, the present study shows that telemetric RTase activity as well as the endogenous non-telomeric RTase activity is also highly expressed in the cancer cell lines, compared with those of normal cell lines. The present results showed that the telemetric RTase activity and endogenous non-telomeric RTase activity were downregulated in the cancer cell lines treated with RTase inhibitors, including EFA. It has been reported that the eukaryotic cells generally display the three main classes of RTase enzyme, including retroviruses, retrotransposons, and telomerase, respectively, and there are some important differences in their function and structure [3]. Based on present results, it is not still unclear whether RTase inhibitors, including EFA, can bind to telomeric RTase enzymes, and inhibit their enzyme activity. The previous study

has shown that the telomeric RTase activity is inhibited by common nucleoside RTase inhibitors using nucleotide analogs, including tenofovir and abacavir, while telomeric RTase is not inhibited with non-nucleoside RTase inhibitors, including NVP and EFA [15, 23]. The nucleotide analogs competitively bind within the catalytic active site of the telomeric RTase activity and inhibit their activity [32]. The inhibition mechanisms of the RTase are different between nucleotide analogs and non-nucleotide analogs that bind at the allosteric site of the enzyme rather than an active site of the enzyme, such as EFA. It is a well-known fact that telomeric RTase activity is commonly decreased in stress-induced senescent as well as replicative senescent cells [5]. Thus, we assumed that the down-regulation of telomeric RTase activity with EFA treatment may be induced by the inhibition of cell growth and cellular senescence, rather than inhibition of their activity via the direct EFA-binding enzyme.

In the present study, inhibition of endogenous RTase activity with EFA treatment, a kind of non-nucleotide RTase inhibitor is induced into delayed cell growth, down-regulation of telomerase activity, and cellular senescence, however, the sensitivity of cellular cytotoxicity against EFA is a slight distinction between cancer cells and normal somatic cells. The more cytotoxicity of EFA was exhibited in the cancer cell lines with high RTase activity than the normal cells with low RTase activity. Otherwise, the arrest of cell growth by RTase inhibition could offer potential cytotoxic chemotherapy in the treatment of cancer cells. In the present study, both non-telomeric endogenous and telomeric RTase activity in the used MSC was displayed at a low level. Theoretically, the constant capacity of in vivo stem cells for unlimited cell growth to self-renewal capacity is controlled by the genetic and epigenetic expression, and interaction and signals from stem-cell niche microenvironment throughout the lifespan, however, the in vitro adult stem cells isolated from stem-cell niche, including MSC are generally exhibited to the limited cell growth and self-renewal capacity [34]. Further, it has been reported that the totipotent embryonic stem cells and pluripotent stem cells displaying unlimited cell growth and self-renewal capacity are tightly associated with high retrotransposon expression [35]. Thus, we assumed that the possibly high endogenous RTase activity in the adult stem cells in vivo is exhibited for unlimited cell growth capacity, however, oral administration of the EFA or other RTase inhibitors for cancer therapy may have some side effects, including cellular cytotoxicity, like the results of the present study. And, the analysis concerning the roles and function of endogenous RTase enzyme was insufficient in the present study, and further detailed studies are necessary for the application of cancer therapy.

The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

References

1. Anwar, S. L., Wulaningsih, W. and Lehmann, U. 2017. Transposable elements in human cancer: causes and consequences of deregulation. *Int. J. Mol. Sci.* **4**, 974.
2. Bayurova, E., Jansons, J., Skrastina, D., Smirnova, O., Mezale, D., Kostyusheva, A., Kostyushev, D., Petkov, S., Podschwadt, P., Valuev-Elliston, V., Sasinovich, S., Korolev, S., Warholm, P., Latanova, A., Starodubova, E., Tukhvatulin, A., Latyshev, O., Selimov, R., Metalnikov, P., Komarov, A., Ivanova, O., Gorodnicheva, T., Kochetkov, S., Gottikh, M., Strumfa, I., Ivanov, A., Gordeychuk, I. and Isaguliants, M. 2019. HIV-1 Reverse transcriptase promotes tumor growth and metastasis formation via ROS-dependent upregulation of twist. *Oxid. Med. Cell Longev.* **2**, 6016278.
3. Belfort, M., Curcio, M. J. and Lue, N. F. 2011. Telomerase and retrotransposons: reverse transcriptases that shaped genomes. *Proc. Natl. Acad. Sci. USA.* **20**, 20304-20310.
4. Bellisai, C., Sciamanna, I., Rovella, P., Giovannini, D., Baranzini, M., Pugliese, G. M., Zeya Ansari, M. S., Milite, C., Sinibaldi-Vallebona, P., Cirilli, R., Sbardella, G., Pichierri, P., Trisciuoglio, D., Lavia, P., Serafino, A. and Spadafora, C. 2020. Reverse transcriptase inhibitors promote the remodelling of nuclear architecture and induce autophagy in prostate cancer cells. *Cancer Lett.* **28**, 133-145.
5. Bernadotte, A., Mikhelson, V. M. and Spivak, I. M. 2016. Markers of cellular senescence. Telomere shortening as a marker of cellular senescence. *Aging (Albany NY)* **8**, 3-11.
6. Carlini, F., Ridolfi, B., Molinari, A., Parisi, C., Bozzuto, G., Toccacieli, L., Formisano, G., De Orsi, D., Paradisi, S., Grober, O. M., Ravo, M., Weisz, A., Arcieri, R., Vella, S. and Gaudi, S. 2010. The reverse transcription inhibitor abacavir shows anticancer activity in prostate cancer cell lines. *PLoS One* **5**, e14221.
7. Childs, B. G., Baker, D. J., Kirkland, J. L., Campisi, J. and van Deursen, J. M. 2014. Senescence and apoptosis: dueling or complementary cell fates? *EMBO Rep.* **15**, 1139-1153.
8. Coffin, J. M. and Fan, H. 2016. The discovery of reverse transcriptase. *Annu. Rev. Virol.* **3**, 29-51
9. Ekram, M. B., Kang, K., Kim, H. and Kim, J. 2017. Retrotransposons as a major source of epigenetic variations in

the mammalian genome. *Epigenetics* **7**, 370-382.
10. Giardini, M. A., Segatto, M., da Silva, M. S., Nunes, V. S. and Cano, M. I. 2014. Telomere and telomerase biology. *Prog. Mol. Biol. Transl. Sci.* **125**, 1-40.
11. Göke, J. and Ng, H. H. 2016. CTRL+INSERT: retrotransposons and their contribution to regulation and innovation of the transcriptome. *EMBO Rep.* **17**, 1131-1144.
12. Grundy, E. E., Diab, N. and Chiappinelli, K. B. 2022. Transposable element regulation and expression in cancer. *FEBS J.* **289**, 1160-1179.
13. Gu, S. X., Lu, H. H., Liu, G. Y., Ju, X. L. and Zhu, Y. Y. 2018. Advances in diarylpyrimidines and related analogues as HIV-1 nonnucleoside reverse transcriptase inhibitors. *Eur. J. Med. Chem.* **158**, 371-392.
14. Hecht, M., Harrer, T., Körber, V., Sarpong, E. O., Moser, F., Fiebig, N., Schwegler, M., Stürzl, M., Fietkau, R. and Distel, L. V. 2018. Cytotoxic effect of Efavirenz in BxPC-3 pancreatic cancer cells is based on oxidative stress and is synergistic with ionizing radiation. *Oncol Lett.* **15**, 1728-1736.
15. Hukezalie, K. R., Thumati, N. R., Côté, H. C. and Wong, J. M. 2012. *In vitro* and *ex vivo* inhibition of human telomerase by anti-HIV nucleoside reverse transcriptase inhibitors (NRTIs) but not by non-NRTIs. *PLoS One* **7**, e47505.
16. Ilango, S., Paital, B., Jayachandran, P., Padma, P. R. and Nirmaladevi, R. 2020. Epigenetic alterations in cancer. *Front. Biosci. (Landmark Ed).* **25**, 1058-1109.
17. Jeon, B. G., Jang, S. J., Park, J. S., Subbara, R. B., Jeong, G. J., Park, B. W. and Rho, G. J. 2015. Differentiation potential of mesenchymal stem cells isolated from human dental tissues into non-mesodermal lineage. *Anim. Cell. Syst.* **19**, 321-331.
18. Jeon, B. G., Kumar, B. M., Kang, E. J., Ock, S. A., Lee, S. L., Kwack, D. O., Byun, J. H., Park, B. W. and Rho, G. J. 2011. Characterization and comparison of telomere length, telomerase and reverse transcriptase activity and gene expression in human mesenchymal stem cells and cancer cells of various origins. *Cell Tissue Res.* **345**, 149-161.
19. Kim, D. Y., Moon, S. H., Han, J. H., Kim, M. J., Oh, S. J., Bharti, D., Lee, S. H., Park, J. K., Rho, G. J. and Jeon, B. G. 2020. Terminal differentiation into adipocyte and growth inhibition by PPARγ activation in human A549 lung adenocarcinoma cells. *Anim. Cell. Syst.* **24**, 329-340.
20. Kong, C. M., Lee, X. W. and Wang, X. 2013. Telomere shortening in human diseases. *FEBS J.* **280**, 3180-3193.
21. Lawson, H. A., Liang, Y. and Wang, T. 2023. Transposable elements in mammalian chromatin organization. *Nat. Rev. Genet.* **24**, 712-723.
22. Lee, W. C., Kim, D. Y., Kim, M. J., Lee, H. J., Bharti, D., Lee, S. H., Kang, Y. H., Rho, G. J. and Jeon, B. G. 2019. Delay of cell growth and loss of stemness by inhibition of reverse transcription in human mesenchymal stem cells derived from dental tissue. *Anim. Cell. Syst.* **23**, 335-345.
23. Leeansyah, E., Cameron, P. U., Solomon, A., Tennakoon, S., Velayudham, P., Gouillou, M., Spelman, T., Hearps, A., Fairley, C., Smit de, V., Pierce, A. B., Armishaw, J., Crowe, S. M., Cooper, D. A., Koelsch, K. K., Liu, J. P., Chuah, J. and Lewin, S. R. 2013. Inhibition of telomerase activity by human immunodeficiency virus (HIV) nucleos(t)ide reverse transcriptase inhibitors: a potential factor contributing to HIV-associated accelerated aging. *J. Infect Dis.* **207**, 1157-1165.
24. Liu, W., Song, X. L., Zhao, S. C., He, M., Wang, H., Chen, Z., Xiang, W., Yi, G., Qi, S. and Liu, Y. 2018. Antitumor activity and mechanism of a reverse transcriptase inhibitor, dapivirine, in glioblastoma. *J. Cancer* **9**, 117-128.
25. Mills, R. E., Bennett, E. A., Iskow, R. C. and Devine, S. E. 2007. Which transposable elements are active in the human genome? *Trends Genet.* **23**, 183-191.
26. Pantzartzi. C. N., Pergner, J. and Kozmik, Z. 2018. The role of transposable elements in functional evolution of amphioxus genome: the case of opsin gene family. *Sci. Rep.* **8**, 2506.
27. Sciamanna, I., De Luca, C. and Spadafora, C. 2016. The Reverse Transcriptase Encoded by LINE-1 Retrotransposons in the genesis, progression, and therapy of cancer. *Front. Chem.* **4**, 6.
28. Sciamanna, I., Sinibaldi-Vallebona, P., Serafino, A. and Spadafora, C. 2018. LINE-1-encoded reverse Transcriptase as a target in cancer therapy. *Front. Biosci.* **23**, 1360-1369.
29. Sciamanna, I., Vitullo, P., Curatolo, A. and Spadafora, C. 2009. Retrotransposons, reverse transcriptase and the genesis of new genetic information. *Gene* **448**, 180-186.
30. Seckler, J. M., Barkley, M. D. and Wintrode, P. L. 2011. Allosteric suppression of HIV-1 reverse transcriptase structural dynamics upon inhibitor binding. *Biophys. J.* **100**, 144-153.
31. Srinivas, N., Rachakonda, S. and Kumar, R. 2020. Telomeres and telomere length: A general overview. *Cancers (Basel),* **12**, 558.
32. Tendian, S. W. and Parker, W. B. 2000. Interaction of deoxyguanosine nucleotide analogs with human telomerase. *Mol. Pharmacol.* **57**, 695-699.
33. Vanangamudi, M., Palaniappan, S., Kathiravan, M. K. and Namasivayam, V. 2023. Strategies in the design and development of non-nucleoside reverse transcriptase inhibitors (NNRTIs). *Viruses* **15**, 1992.
34. Voog, J. and Jones, D. L. 2010. Stem cells and the niche: a dynamic duo. *Cell Stem Cell* **6**, 103-115.
35. Wang, J., Huang, J. and Shi, G. 2020. Retrotransposons in pluripotent stem cells. *Cell Regen.* **9**, 4.
36. Wu, J., Wu, C., Xing, F., Cao, L., Zeng, W., Guo, L., Li, P., Zhong, Y., Jiang, H., Luo, M., Shi, G., Bu, L., Ji, Y., Hou, P., Peng, H., Huang, J., Li, C. and Guo, D. 2021. Endogenous reverse transcriptase and RNase H-mediated antiviral mechanism in embryonic stem cells. *Cell Res.* **31**, 998-1010.

초록 : 사람의 암세포주 및 정상세포주에서 역전사 효소의 억제에 의한 세포 성장의 제한

김미정[1] · 이성호[2] · 박종근[3] · 전병균[1]*
([1]경상대학교 생물교육과, [2]경상대학교 생명과학부, [3]경상대학교 화학교육과)

이 연구는 여러 종류의 암세포주(A-549, AGS, HCT-116, MDA-MB-231 및 U 87-MG)와 정상세포주(MRC-5 및 MSC)에 RNA를 DNA로 전환시킬 수 있는 역전사 효소의 억제 처리 후 세포의 성장에 미치는 영향을 비교 조사하였다. 각 세포주에 efavirenz (EFA) 역전사 효소 억제제를 1주일 동안 처리하였을 때, 세포 성장의 반억제농도(IC_{50}) 값은 암세포주보다 정상세포주에서 더 높은 값을 나타냈다. 결정된 IC_{50} 값에 따라 15 μM 농도로 EFA를 1주일 동안 처리하였을 때, 역전사 효소 및 말단소립 복원 효소의 활성은 EFA 처리군에서 비처리군에 비하여 유의적으로($p<0.05$) 감소하였다. 그러나, 역전사 효소 및 말단소립 복원 효소의 활성은 정상세포주에서는 검출되지 않았다. 15 μM EFA를 처리한 후, 암세포주와 정상세포주에서 세포성장율을 비교하였을 때, EFA 처리는 모든 세포의 성장을 억제하였는데, 정상세포주보다 암세포주에서 세포의 성장율이 현저하게($p<0.05$) 감소하였다. 또한, 역전사 효소의 억제가 세포의 노화 및 사멸에 미치는 영향을 분석하기 위해 노화 관련 ß-galactosidase 효소의 활성을 분석하였을 때, EFA 처리가 노화 관련 효소 활성이 점점 증가하는 것으로 보아, EFA 역전사 효소의 처리는 세포 노화 및 세포 사멸을 유도하는 것을 알 수 있었다. 이상의 결과를 바탕으로, 역전사 효소의 억제는 정상세포보다는 암세포의 성장을 더 억제하는 것을 알 수 있었지만, 항암치료 등에서 응용되기 위해서는 세포에서 역전사 효소의 기능과 역할에 대해서는 좀 더 심도있는 연구가 필요할 것으로 생각된다.