8-Chloro-Adenosine Inhibits Proliferation of MDA-MB-231 and SK-BR-3 Breast Cancer Cells by Regulating ADAR1/p53 Signaling Pathway

Hong-Yue Ding1,*, Wan-Yong Yang2,*, Li-Hong Zhang1,*, Li Li1, Feng Xie1, Hua-Yi Li1, Xiao-Yu Chen1, Zeng Tu1, Yi Li1, Yong Chen3, and Sheng-Yong Yang1

Abstract
8-Chloro-adenosine (8-Cl-Ado) has been shown to exhibit its antitumor activity by inducing apoptosis in human lung cancer A549 and H1299 cells or autophagy in chronic lymphocytic leukemia, and MDA-MB-231 and MCF-7 breast cancer cells. Adenosine deaminases acting on RNA 1 (ADAR1) is tightly associated with cancer development and progression. The aim of this study was to investigate the role of ADAR1 in the proliferation of MDA-MB-231 and SK-BR-3 breast cancer cell lines after 8-Cl-Ado exposure and its possible mechanisms. After 8-Cl-Ado exposure, CCK-8 assay was performed to determine the cell proliferation; flow cytometry was used to analyze the cell cycle profiles and apoptosis; and the protein levels of ADAR1, p53, p21, and cyclin D1 were measured by western blotting. The results showed that the cell proliferation was greatly inhibited, G1 cell cycle was arrested, and apoptosis was induced after 8-Cl-Ado exposure. ADAR1 and cyclin D1 protein levels were dramatically decreased, while p53 and p21 levels were increased after 8-Cl-Ado exposure. Moreover, the cell growth inhibition was rescued, apoptosis was reduced, and p53 and p21 protein levels were downregulated, while cyclin D1 was upregulated when cells were transfected with plasmids expressing ADAR1 proteins. More importantly, RNA-binding domain of ADAR1 is critical to the cell growth inhibition of breast cancer cells exposed to 8-Cl-Ado. Together, 8-Cl-Ado inhibits the cell proliferation, induces G1 phase arrest and apoptosis at least by targeting ADAR1/p53/p21 signaling pathway. The findings may provide us with insights into the role of ADAR1 in breast cancer progression and help us better understand the effects of 8-Cl-Ado in the treatment of breast cancer.

Keywords
8-Cl-Ado, breast cancer cells, cell cycle, ADAR1, double-stranded RNA-binding domain

1 Department of Biochemistry and Molecular Biology, Molecular Medicine and Cancer Research Center, Chongqing Medical University, Chongqing, China
2 Dongguan Waterfront Zone Central Hospital, Dongguan, Guangdong, China
3 Department of Radiology and Intervention, The General Hospital of Ningxia Medical University, Yinchuan, China
* These authors contributed equally to this article

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Corresponding Authors:
Yong Chen, Department of Radiology and Intervention, The General Hospital of Ningxia Medical University, Yinchuan 750004, China.
Email: chenyong6981@sina.com

Yi Li and Sheng-Yong Yang, Department of Biochemistry and Molecular Biology, Molecular Medicine and Cancer Research Center, Chongqing Medical University, Chongqing 400016, China.
Emails: Liyi@cqmu.edu.cn or cecilylee@126.com; yangshengyong@cqmu.edu.cn
Introduction

Breast cancer is one of the most prevalent malignant tumors and has become the major cause of cancer deaths in women worldwide. Although improvements in therapies, including surgery, radiotherapy, chemotherapy, immunotherapy, and targeted therapy, have led to decreased mortality in recent years, the prognosis of patients diagnosed with advanced breast cancer remains disappointing, therapy side effects, and resistance persist considerable clinical issues. There-

Materials and Methods

Cell Culture and Treatment

The human breast cancer cell lines MDA-MB-231 and SK-BR-3 were cultured under 5% CO₂ at 37°C in Roswell Park Memorial Institute 1640 medium (RPMI-1640, HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (PAN-Biotech, Adenbach, Germany). 8-Cl-Ado (BIOLOG life Science Institute, Bremen, Germany) was dissolved in 0.9% NaCl solution at the desired concentration.

Plasmids and Cell Transfection

The plasmids for ADAR1-p150, ADAR1-p110, ADAR1-ΔE/A, and ADAR1-ΔR were gifts from professor Qingde Wang from University of Pittsburgh. The MDA-MB-231 and SK-BR-3 cells were transfected with 3 μg of different kinds of ADAR1 plasmids using Lipofecta-

Cell Proliferation Assays

As described previously, briefly, cells were seeded at a density of 3 × 10⁵ cells per well in 96-well plates, with three wells per group. After 24 h, different concentrations of 8-Cl-Ado were added to wells, and the cells were further cultured under normal culture conditions for different time points. Then 10 μl CCK-8 solution (Boster, Wuhan, China) was added into each well and incubated for half an hour. The absorbance was measured at an optical density of 450 nm using microplate reader (Bio-Rad, Hercules, CA, USA). The experiment was repeated at least three times.

Colony Formation Assay

MDA-MB-231 cells and SK-Br-3 cells were seeded in a six-well plate at a density of 500 cells per well in triplicate. After 24 h, the cells were exposed to 10 μM 8-Cl-Ado for 10 days. Then, the cells were washed with PBS, fixed in 4% formaldehyde, and stained with 0.1% crystal violet for 20 min at room temperature. The number of colonies with at least 50 cells was counted. Colony formation rate was calculated by dividing the number of colonies after 10 days by the number of cells seeded initially.

Flow Cytometric Analysis

As described previously for the cell cycle analysis, briefly, cells were seeded at a density of 2.5 × 10⁵ cells per well in six-well plates. After 24 h, 8-Cl-Ado was added to plates, and cells were further cultured for different time points. Cells were harvested, fixed with 1 ml of ice-cold 70% ethanol, incubated at 4°C for 6 h, and then analyzed by using FACS Calibur system (BD company, Franklin Lakes, NJ,

RNA editing is tightly associated with the development of a variety of malignancies. A-to-I RNA editing, catalyzed by adenosine deaminases acting on RNA (ADARs) through converting adenosine into inosine at a specific site, is an eventful post-transcriptional modification in mammals. Inosine is read as guanosine by translation machinery, resulting in codon changes, alternative splicing or affect targeting, and maturation of micro-

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USA). The cells labeling annexin V and propidium iodide were also determined by using the same FACS Calibur system, as previously reported. All experiments were performed at least three times.

**Western Blotting**

Cell lysates were separated on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and shifted onto nitrocellulose membranes. Then, the nitrocellulose membranes were incubated with primary antibodies overnight at 4°C, and the secondary antibodies for 1.5 h at room temperature. Bands were visualized by an ECL chemiluminescent detection system (Thermo Fisher Scientific, Rochester, NY, USA). The blots were screened with the software Quantity One (Bio-Rad) and normalized against actin level. Anti-ADAR1 antibody was acquired from Abcam company (Cambridge, MA, USA), and anti-β-actin, anti-p53, anti-p21, and anti-cyclin D1 antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA).

**Statistical Analysis**

All experiments were performed in triplicate. The data are expressed as the mean ± standard deviation and analyzed using the software: GraphPad Prism 5.02 (GraphPad, San Diego, CA, USA) and the SPSS 16.0 software (SPSS, Chicago, IL, USA). P-value of <0.05 was considered as statistical significance.

**Results**

8-Cl-Ado Inhibits Proliferation of Breast Cancer Cell Growth

MDA-MB-231 and SK-BR-3 breast cancer cell lines were exposed to different concentrations of 8-Cl-Ado (0.01, 0.1, 0.3, 1, 10, 30, and 100 μM) for 96 h or exposed to 10 μM of 8-Cl-Ado for indicated time points. Then, the CCK8 assay was performed to detect growth inhibition. As shown in Fig. 1A, B, cell growth was inhibited by 8-Cl-Ado in both dose- and time-dependent manners in both cell lines with an IC50 for growth inhibition of 0.52 and 1.4 μM in the MDA-MB-231 and SK-BR-3 cells, respectively. In addition, the inhibition rate reached about 80% at the concentration of 10 μM 8-Cl-Ado in MDA-MB-231 cells and 90% in SK-BR-3 cells at 96 h. Moreover, 8-Cl-Ado inhibited colony formation of breast cancer cells (Fig. 1C, D). The colony formation rate of MDA-MB-231 and SK-BR-3 cells was reduced by about 40% and 30% after 10 μM of 8-Cl-Ado exposure for 10 days, respectively, compared to that of corresponding control group.

8-Cl-Ado Induces Both G1 Cell Cycle Arrest and Apoptosis of Breast Cancer Cells

To determine whether the growth inhibition of breast cancer cells by 8-Cl-Ado is due to cytostatic activity and/or an
apoptotic response, MDA-MB-231 and SK-BR-3 cells were exposed to 10 μM 8-Cl-Ado for different time points, and flow cytometry was performed to assess their cell cycle profile and apoptotic rate. As shown in Fig. 1E, G, after 8-Cl-Ado exposure, percentage of G1 subpopulation was significantly increased from 55.51% to 73.78% within 24–72 h, while percentage of S subpopulation was decreased from 38.28% to 20.51%; however, percentage of G2/M subpopulation was unaltered in MDA-MB-231 cells. Further, annexin V and propidium iodide–positive cells were increased from 15% to 52% after 10 μM 8-Cl-Ado exposure in MDA-MB-231 cells in a time-dependent manner (Fig. II, K). Similar result and trend for cell cycle profile and percentages of annexin V and propidium iodine–positive cells was observed from SK-BR-3 cells (Fig. 1F, H, J, and L), indicating that 8-Cl-Ado-induced G1 cell cycle arrest and apoptosis in breast cancer cells. These results suggest that the growth inhibition of breast cancer cells by 8-Cl-Ado was due to both cytostatic activity and/or apoptosis.

8-Cl-Ado Downregulates ADAR1 Protein Levels in Breast Cancer Cells

Next, we want to know if the cell growth inhibition caused by 8-Cl-Ado was associated with RNA-editing enzyme ADAR1, so we detected the expression level of ADAR1 protein using Western blot assay after breast cancer cells were exposed to various concentrations of 8-Cl-Ado for 48 h. As shown in Fig. 2, both ADAR1-p150 and ADAR1-p110 protein levels were dramatically reduced in MDA-MB-231 (Fig. 2A) and SK-BR-3 (Fig. 2B) cells in a dose-dependent manner, suggesting that 8-Cl-Ado may inhibit cell growth through downregulating ADAR1 protein expression.

ADAR1 Inhibits p53/p21 Signaling Pathway in Breast Cancer Cells

To determine whether and how cell growth inhibition was caused by reduced ADAR1 protein levels, Western blots for ADAR1, p53, p21, and cyclin D1 proteins were performed after cells were exposed to 10 μM 8-Cl-Ado for 12–72 h. As shown in Fig. 2, protein expression levels of ADAR1 and cyclin D1 were significantly reduced, whereas p53 and p21 protein levels were significantly increased in both MDA-MB-231 and SK-BR-3 cells (Fig. 2C, Supplemental Fig. S1) in a time-dependent manner. When these two cell lines were overexpressed with either ADAR1-p150 or ADAR1-p110 proteins by transfecting plasmids expressing ADAR1 proteins, as expected, p53 and p21 protein levels were decreased, while cyclin D1 increased after 8-Cl-Ado exposure (Fig. 2D, Supplemental Fig. S1). In addition, the cell growth inhibition was rescued (Fig. 3A, B) and the percentages of the cells labeling annexin V and propidium iodide were significantly decreased (Fig. 3C, D) after overexpressing either ADAR1-110 or ADAR1-150 protein in the cells exposed to 8-Cl-Ado. These results indicate that ADAR1 could inhibit p53/p21 signaling pathway, which may contribute to G1 cell cycle arrest and apoptosis of breast cancer cells exposed to 8-Cl-Ado.

The RNA-Binding Domain of ADAR1 Plays an Important Role in 8-Cl-Ado-Induced G1 Cell Cycle Arrest of Breast Cancer Cell

To determine which domain (or region) of ADAR1 is critical to G1 cell cycle arrest in breast cancer cells, different types of ADAR1 plasmids were transfected into breast cancer cells (Fig. 4A, B), then exposed to 10 μM 8-Cl-Ado for 48 h, and cell cycle profile was analyzed by performing flow cytometry. As shown in Fig. 4, the percentage of G1 phase in wild-type ADAR1 plasmid groups (ADAR1-p150 and ADAR1-p110) and the ADAR1-ΔE/A group in MDA-MB-231 cells (Fig. 4C) and SK-BR-3 cells (Fig. 4D) was remarkably reduced, compared to the empty vector control group, but no significant difference was observed in the ADAR1-ΔR plasmid group lacking RNA-binding domain, suggesting that the RNA-binding domain of ADAR1 plays an important role in 8-Cl-Ado-induced G1 phase arrest of breast cancer cells.

Discussion

In this study, we found that 8-Cl-Ado inhibits cell growth by inducing G1 cell cycle arrest and apoptosis of breast cancer cells. Mechanismly, 8-Cl-Ado activates p53/p21 signaling pathway at least by downregulating expression level of RNA-editing enzyme ADAR1 protein, leading to growth inhibition of breast cancer cells. Moreover, we demonstrated that RNA-binding domain but not RNA-editing activity of ADAR1 plays a very important role in this process. The results may shed light on the mechanisms of 8-Cl-Ado action in the treatment of breast cancer.

It has been reported that 8-Cl-Ado exhibits its antitumor activity by inducing apoptosis or autophagy in some types of cancer cell lines, including lung cancer cells, leukemia cells, and breast cancer cells. In the present study, we found that 8-Cl-Ado inhibited the breast cancer cell growth by inducing G1 cell cycle arrest and apoptosis at least through downregulating ADAR1 proteins in MDA-MB-231 and SK-BR-3 cells, which is consistent with our previous findings that 8-Cl-Ado downregulates ADAR1 and inhibits cell growth of breast cancer cell lines. In our previous study, we did not detect effects of 8-Cl-Ado on cell cycle profile and apoptosis in the cell lines. While in Stellrecht’s study, 8-Cl-Ado does not significantly change the percentages of G1 or S phase of the cell cycle of breast cancer cell lines during three-day incubation. This may due to different cell lines used or the usage of 8-Cl-Ado from different sources for experiments. Whether and how ADAR1 causes changes of the cell cycle profile or apoptosis is not very clear in breast cancer cells after 8-Cl-Ado exposure.
Figure 2. Protein levels of ADAR1, p53, p21, and cyclin D1 after 8-Cl-Ado exposure in breast cancer cells with or without overexpression of ADAR1. (A, B) ADAR1 protein expression levels were detected by Western blotting in MDA-MB-231 and SK-BR-3 breast cancer cells exposed to various concentrations of 8-Cl-Ado for 48 h. The relative levels of ADAR1-p150 and ADAR1-p110 in Western blotting were quantified (bottom of A and B). The ratio of ADAR1/β-actin proteins in control cells was normalized to “1.” (C) Expression levels of ADAR1, p53, p21, and cyclin D1 protein by Western blotting in MDA-MB-231 and SK-BR-3 breast cancer cells exposed to 10 μM 8-Cl-Ado for 12–72 h. (D) Expression levels of ADAR1, p53, p21, and cyclin D1 protein by Western blotting in ADAR1 overexpressed MDA-MB-231 and SK-BR-3 breast cancer cells exposed to 10 μM 8-Cl-Ado for 48 h. The numbers below the bands show the relative levels of proteins. Also see Supplemental Fig. S1 for analysis of protein expression levels. 8-Cl-Ado: 8-chloro-adenosine; ADAR1: adenosine deaminases acting on RNA 1.
ADAR1, a type of RNA-editing enzyme that catalyzes the adenosine into inosine on double-stranded RNA, is highly expressed in breast, lung, liver, and esophageal cancer, as well as in chronic myelogenous leukemia, where it promotes tumor progression depending on cancer types\textsuperscript{17–21}. Previously, we showed that ADAR1 is increased in both breast cancer tissue samples and breast cancer cell line, especially, ADAR1-p110 isoform is dominantly expressed and promotes the proliferation and migration of breast cancer cells\textsuperscript{7,8}. In the present study, ADAR1 protein levels were dramatically reduced after 8-Cl-Ado exposure in both dose- and time-dependent manners in breast cancer cell lines, suggesting that 8-Cl-Ado may mainly target ADAR1 to inhibit cell growth. Indeed, cell growth inhibition was reduced after 8-Cl-Ado exposure when ADAR1 proteins were overexpressed by transfecting ADAR1 expression plasmids and treated with 10 μM 8-Cl-Ado for 48 h, then CCK-8 assay (A and B) and flow cytometric analysis (C and D) were performed. The experiments were repeated at least three times, and the data are expressed as the mean ± SD; **p < 0.01, ***p < 0.001. 8-Cl-Ado: 8-chloro-adenosine; ADAR1: adenosine deaminases acting on RNA 1; SD: standard deviation.

![Figure 3](image)

**Figure 3**. Effects of overexpression of ADAR1 on cell proliferation and apoptosis of breast cancer cells. MDA-MB-231 and SK-BR-3 breast cancer cells were overexpressed ADAR1 proteins by transfecting ADAR1 expression plasmids and treated with 10 μM 8-Cl-Ado for 48 h, then CCK-8 assay (A and B) and flow cytometric analysis (C and D) were performed. The experiments were repeated at least three times, and the data are expressed as the mean ± SD; **p < 0.01, ***p < 0.001. 8-Cl-Ado: 8-chloro-adenosine; ADAR1: adenosine deaminases acting on RNA 1; SD: standard deviation.

A growing body of evidence suggested that A-to-I RNA editing mediated by ADAR1 is involved in cancer development and progression\textsuperscript{26–29}. It has been reported that the editing frequency of global transcripts mediated by ADAR1 was higher in breast tumors than in normal tissues\textsuperscript{30}. Although ADAR1 is an RNA-editing enzyme, it also functions independently of editing activity in some important biological or pathological process\textsuperscript{15}. However, in the present study, we demonstrated that RNA-binding domain but not editing activity of ADAR1 plays a critical role in G1 phase arrest of the cell cycle after 8-Cl-Ado treatment by overexpressing wild-type ADAR1, mutant ADAR1 (E912A, an inactive form of ADAR1 for RNA editing)\textsuperscript{22}, and truncated ADAR1 protein that is lacking three RNA-binding domains\textsuperscript{23} in the cells, respectively. The mechanisms underlying this need to be further investigated.

Usually, cancer cells often exhibit dysregulation, abnormalities, and cumulative mutations of the cell cycle, leading to genomic instability and abnormal proliferation\textsuperscript{31–33}. Therefore, the destruction of cancer cell cycle progression is an important target for cancer therapy. Some studies reported that 8-Cl-Ado inhibits growth of human lung cancer cells by inducing G2/M arrest and mitotic catastrophe\textsuperscript{34}. Also, 8-Cl-Ado induces S arrest in human myelocytic leukemia
K562 cells\textsuperscript{35}. Remarkably, our current study provides evidence that 8-Cl-Ado induced G1 phase arrest and apoptosis of MDA-MB-231 and SK-BR-3 breast cancer cells and changed the levels of cyclin-related proteins (P53, P21, and cyclin D1) by downregulating ADAR1. The transcription factor p53, a well-known tumor suppressor, plays an important role in cell cycle arrest\textsuperscript{36}. p53 controls the cell cycle progression by transactivating the components of key downstream, including p21, cyclin D1, and other molecules involved in the cell cycle regulation\textsuperscript{37}. Activation of p53 can exhibit the role of its antitumor as a tumor suppressor by activating transcription of various target genes such as p21 and p27\textsuperscript{38,39}. p21 belongs to the Cip/Kip family of CDK inhibitors that inhibit the activity of cyclin/CDK complexes such as cyclin E/CDK2, thereby ultimately preventing cell cycle at G1-S phase or inducing apoptosis\textsuperscript{40–42}. Our findings show that ADAR1 was negatively correlated with P53, and ADAR1 protein level was reduced after 8-Cl-Ado exposure, which upregulates p53 to induce G1 arrest or probably apoptosis in breast cancer cells.

It should be noted that inhibition of p53/p21 signaling pathway by ADAR1 is not the only way to induce G1 cell cycle arrest or apoptosis of breast cancer cells exposed to 8-Cl-Ado. It is well known that ADAR1 impacts proliferation and apoptosis\textsuperscript{43–45}, and it is widely believed that the effect is mediated by dsRNA accumulation (which ADAR inhibits) and the subsequent activation of the interferon response\textsuperscript{22,45}. Indeed, α- and β-interferon levels were increased by real-time polymerase chain reaction after 8-Cl-Ado exposure in both MDA-MB-231 and SK-BR-3 cells (data not shown), which also contributes to inhibition of the breast cancer cells by 8-Cl-Ado. Further, it is reported that ADAR1 affects tumorigenesis by editing microRNA itself or microRNA-binding site. We recently showed that 8-Cl-Ado inhibits proliferation of MDA-MB-231 and MCF-7 breast cancer cell lines through ADAR1-regulated miRNA 335-5p\textsuperscript{8}, indicating that microRNAs controlled by ADAR1 are involved in actions of 8-Cl-Ado in breast cancer cells. In addition, 8-Cl-Ado also exhibits its antitumor activity by inducing autophagy in MCF-7 and BT-474 breast cancer cells\textsuperscript{6}. RNA-seq, microRNA array and lncRNA array, or m6A-MeRIP assay should be performed to investigate the other possibilities and the details about actions of 8-Cl-Ado in breast cancer cells in the future.

In summary, our findings provide that 8-Cl-Ado inhibits the cell proliferation by inducing G1 phase arrest and apoptosis of breast cancer cells at least by downregulating ADAR1, and its mechanism may relate to inhibition of p53 by ADAR1, and the RNA-binding domain of ADAR1 plays a critical role in this process. However, how 8-Cl-Ado downregulates ADAR1 and how ADAR1 inhibits P53 through the RNA-binding domain of ADAR1 need to be further studied in the future. Therefore, the identification of targets of 8-Cl-Ado and ADAR1-interacting partners will provide more mechanistic insights into the role of ADAR1 in breast cancer progression.

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Ethical Approval

This study was approved by Ethics Committee of Chongqing Medical University, Chongqing, China.
Statement of Human and Animal Rights
This article does not contain any studies with human or animal subjects.

Statement of Informed Consent
We confirm that there are no human subjects in this article and informed consent is not applicable.

Declaration of Conflicting Interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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ORCID iD
Sheng-Yong Yang https://orcid.org/0000-0001-7312-0255

Supplemental Material
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