

# Effect of Nonequilibrium Atmospheric Pressure Plasma on Cancer-Initiating Cells

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**ABSTRACT:** Medical applications of nonequilibrium atmospheric pressure plasma (NEAPP) have been reported in cancer therapy. Cells with tumorigenic potential are limited to a small population, called cancer-initiating cells (CICs), which are usually expressing aldehyde dehydrogenase (ALDH) in a high level. CICs are believed to cause recurrence or metastasis due to their resistance to apoptosis. Here, we examined the effect of NEAPP on CICs using human uterine endometrioid adenocarcinoma cells and poorly differentiated human gastric carcinoma cells. When treated with NEAPP, ALDH-high cells fell into apoptosis in a comparable level to ALDH-low cells. These results suggested that NEAPP treatment was effective not only on non-CICs but also on CICs. NEAPP might become a new therapeutic approach to CICs.

**KEY WORDS:** nonequilibrium atmospheric pressure plasma; cancer-initiating cells; aldehyde dehydrogenase

## I. INTRODUCTION

Recently, medical applications of nonequilibrium atmospheric pressure plasma (NEAPP), also known as cold plasma or nonthermal plasma, have been reported in cancer therapy.<sup>1-6</sup> Tumor cells with tumorigenic potential are limited to a small population, called cancer-initiating cells (CICs), in several tumors, such as leukemia, breast, brain, and colon cancers.<sup>7-14</sup> CICs efficiently efflux antitumor chemicals, resist radiotherapy, and are believed to cause cancer recurrence or metastasis. Recent studies have revealed high aldehyde dehydrogenase (ALDH) activity as a characteristic of CICs.<sup>15-22</sup> In fact, clinical cases of uterine endometrioid adenocarcinoma with abundant ALDH high-expressing cells showed a poor prognosis.<sup>22</sup> To our knowledge, no studies have been done about the effect of NEAPP treatment on CICs. Therefore, using ALDH as a marker of CICs, we examined the effect of NEAPP on CICs of human uterine endometrioid adenocarcinoma cells (HEC-1) and poorly differentiated human gastric carcinoma cells (GCIY).

## II. MATERIALS AND METHODS

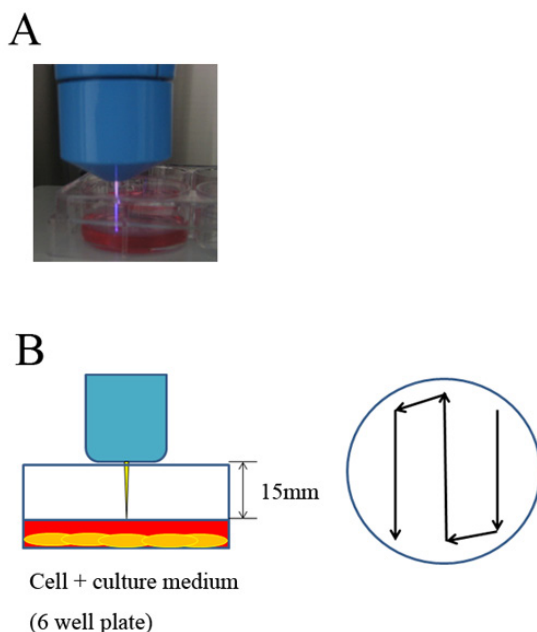
### A. Cell Lines

Human uterine endometrioid adenocarcinoma cell line HEC-1 was obtained from the Health Science Research Resource Bank of Osaka, Japan. Poorly differentiated human

gastric carcinoma cell line GCIY was gifted from Dr. Hayao Nakanishi (Aichi Cancer Center, Japan). Both cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Wako, Osaka, Japan) supplemented with 10% FCS (Nippon Bio-Supply Center, Tokyo, Japan).

## B. Experimental Setup of Nonequilibrium Atmospheric Pressure Plasma (NEAPP)

Cell lines were treated with NEAPP provided from the National Institute of Advanced Industrial Science and Technology (AIST) (Fig. 1A).<sup>23,24</sup> The flow rate of helium gas was set at 2 standard liters/min (slm), and the distance between the plasma source and the samples was fixed at  $L=15$  mm (Fig. 1B). The depth of the medium was 4 mm. Cells were plated at  $3 \times 10^5$  cells in 3 ml medium in a 6-well plate and at  $1 \times 10^5$  cells in 1 ml medium in a 12-well plate. Exposure times of NEAPP treatment were 0, 3, 5, and 10 min. Cells were cultured in plasma-irradiated medium for 20 h after treatment with NEAPP before the first medium change. ALDH-high and -low cells were used for the assay immediately after sorting by flow cytometer.



**FIG. 1:** Nonequilibrium atmospheric pressure plasma (NEAPP) device. (A) NEAPP device from AIST. (B) Schematic diagram of experiment

### C. Cell Viability Assay

Cells treated with NEAPP were plated in 100  $\mu$ L medium in a 96-well plate. On the following day, cell viability was assessed with Premix WST-1 Cell Proliferative Assay System (Takara Bio Inc., Kyoto, Japan) according to the manufacturer's instructions. The absorbance of NEAPP-treated cells at 450 nm was subtracted from the background absorbance (600 nm). The value was divided by that of cells without treatment of NEAPP, and the results are shown as the viability index.

### D. Aldefluor Assay and Isolation of ALDH-high and -low Cells

To check and isolate the cell population with high ALDH activity, the Aldefluor kit (Stem Cell Technologies, Vancouver, BC, Canada) was used according to the manufacturer's instructions and previous reports. Briefly, cells were suspended in Aldefluor assay buffer containing ALDH substrate and BODIPY-aminoacetaldehyde (BAAA). The BAAA was taken up by living cells and converted by intracellular ALDH into BODIPY-aminoacetate, which yields bright fluorescence. The brightly fluorescent ALDH expressing cells were detected with a FACS Canto II or FACS Aria II (BD Biosciences, Franklin Lakes, NJ, USA). As a negative control, cells were stained under identical conditions in the presence of the specific ALDH inhibitor diethylaminobenzaldehyde (DEAB) (Sigma, St. Louis, MO). Data were analyzed by Cell Quest software (BD Biosciences). In HEC-1 and GCIY cell lines, cells with bright and faint fluorescence were defined as ALDH-high and ALDH-low, respectively.

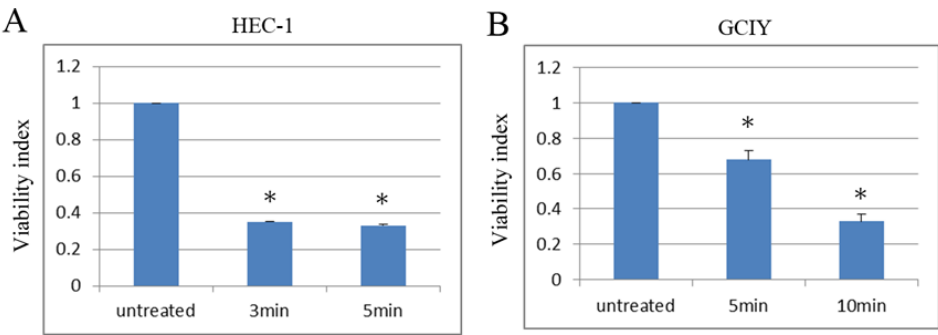
### E. Statistical Analysis

Statistical analyses for experimental studies were performed using Student's *t*-tests. The values are shown as the mean $\pm$ SE of at least three experiments. The *P* values of less than 0.05 were considered to be statistically significant.

## III. RESULTS

### A. Effect of NEAPP on Cancer Cell Lines

The treatment of NEAPP for 3 min reduced the viability of HEC-1 to 30%, and the treatment for 5 min also affected HEC-1 in a similar manner (Fig. 2A), which was consistent with the recent report on the anticancer effect of NEAPP. The viability index of GCIY gastric adenocarcinoma cells was also reduced to approximately 70% by treatment of



**FIG. 2:** Effect of NEAPP on cancer cells. HEC-1 and GCIY cells were plated at a density of  $3 \times 10^5$  in 3 ml DMEM with 10% FCS in a 6-well plate. Exposed times of NEAPP treatment were 0, 3, and 5 min in HEC-1 cells, and 0, 5, 10 min in GCIY cells. On the following day, cell viability was examined using the WST-1 assay. (A) HEC-1 and (B) GCIY cells treated with NEAPP. The values are the means $\pm$ SE of three experiments. \* $p < 0.001$  (Student’s *t*-test)

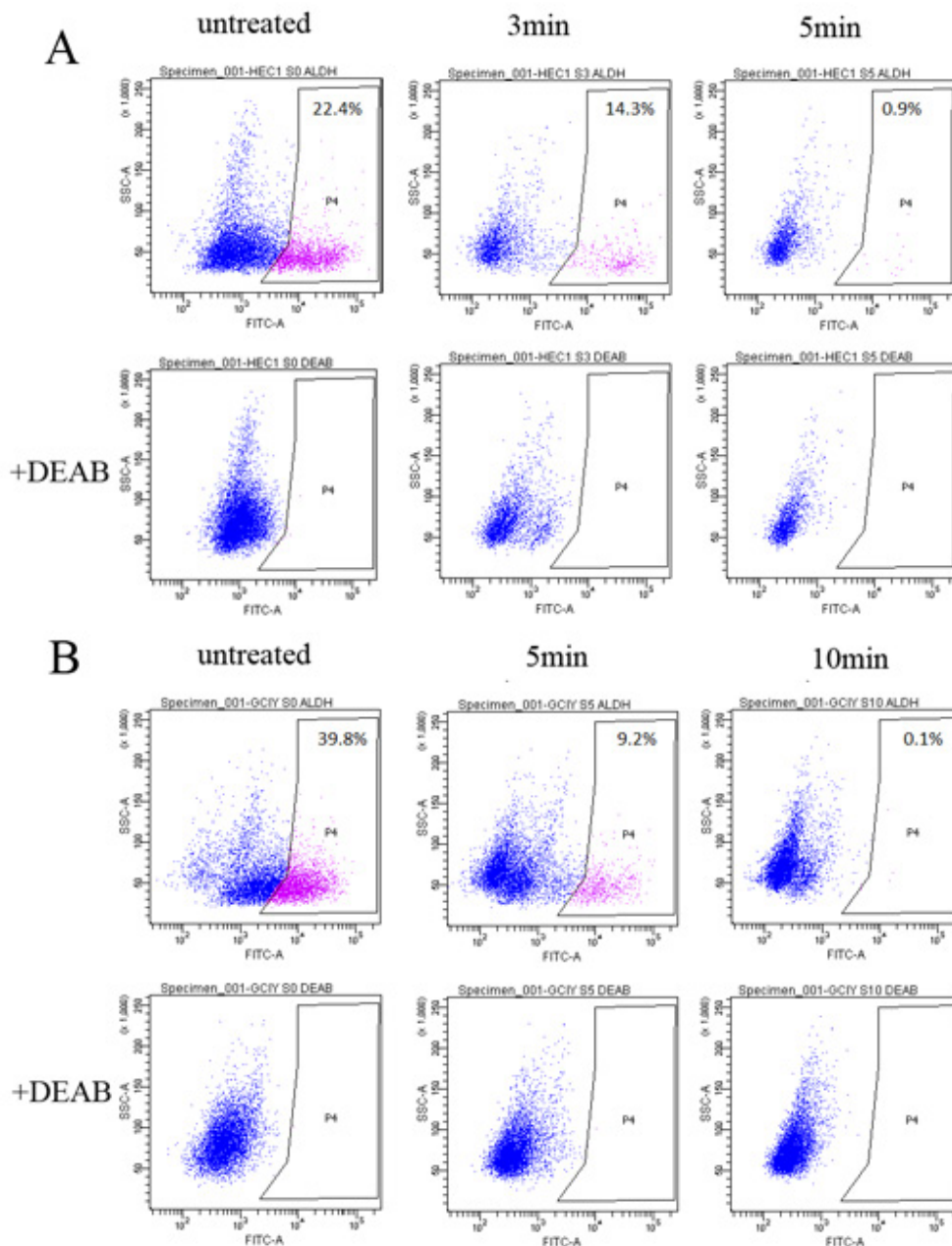
NEAPP for 5 min and 30% for 10 min (Fig. 2B). NEAPP appeared to kill HEC-1 cells more efficiently than GCIY cells.

**B. Effect of NEAPP on ALDH Activity**

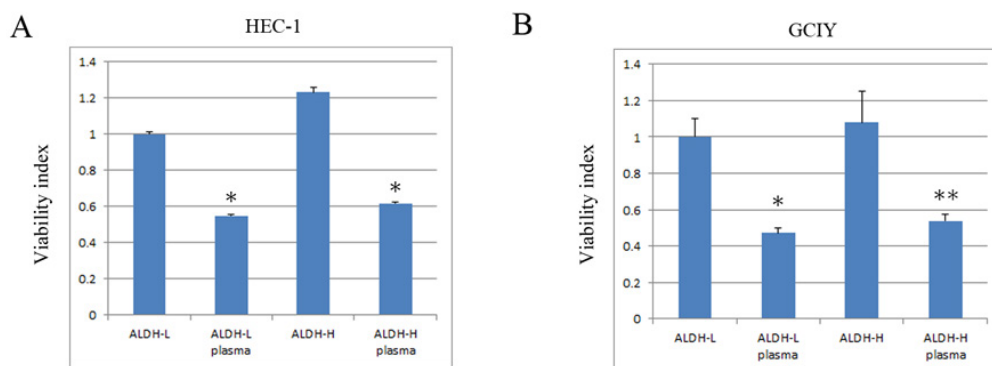
ALDH was recognized as a CIC marker in many types of tumors.<sup>15–22</sup> To examine the changes of ALDH activity after NEAPP treatment, an Aldefluor assay was performed in HEC-1 and GCIY cells. In HEC-1 cells, the treatment of NEAPP for 3 min reduced the amount of ALDH-high population from 22.4% to 14.3%, and the treatment for 5 min reduced it to 0.9% (Fig. 3A). Similarly, in GCIY cells, the amount of ALDH-high population was reduced from 39.8% to 9.2% (exposure time, 5 min) and 0.1% (exposure time, 10 min) after NEAPP treatment (Fig. 3B).

**C. Effect of NEAPP on the Sorted ALDH-high and -low Cells**

To observe the effect of NEAPP more precisely, ALDH-high and ALDH-low cells were sorted by flow cytometer, and treated with NEAPP. In HEC-1 cells, the treatment of NEAPP reduced the viability of sorted ALDH-low cells to approximately 50% (Fig. 4A). The viability by NEAPP was also reduced to half in the sorted ALDH-high cells (Fig. 4A). In GCIY cells, the treatment of NEAPP reduced the viability, and the reduction ratio of ALDH-high cells was comparable to that of ALDH-low cells (Fig. 4B). As ALDH-high populations have the potential of CICs, these results showed that NEAPP treatment affected not only non-CICs but also CICs.



**FIG. 3:** Effect of NEAPP on ALDH activity. Dot-plot of Aldefluor assay without inhibitor is shown on the upper side and that with inhibitor (DEAB) on the lower side. (A) HEC-1 cells treated with NEAPP for 0, 3, and 5 min. (B) GCIY treated with NEAPP for 0, 5, and 10 min. Aldefluor assay was performed on the following day after NEAPP treatment



**FIG. 4:** Effect of NEAPP on ALDH-high and -low cells. NEAPP treatment was performed in the sorted ALDH-high and -low cells. Cell viability was shown in HEC-1 (A) and GCIY (B). The values are the means $\pm$ SE of three experiments. \* $p$ <0.001; \*\* $p$ <0.01 (Student's  $t$ -test)

## IV. DISCUSSION

Since NEAPP was developed, effects of normal or tumor cells, sterilization, and blood coagulation without heating have been reported.<sup>1–6,23,24</sup> Recently, some studies showed that treatment of NEAPP could have an anticancer effect on several types of tumor, such as ovarian cancer, brain tumor, and colon cancer.<sup>1–6</sup> As a mechanism of the anticancer effect, it is considered that electrons, ions, and radicals that occurred due to the NEAPP treatment caused the cells to induce reactive oxygen species and be killed by DNA damage. In the present study, we demonstrated that NEAPP has an antitumor effect on human endometrioid adenocarcinoma and gastric adenocarcinoma cell lines. These results suggest that NEAPP treatment induced an antitumor effect on many types of tumor cells.

Tumors consist of heterogeneous cell populations derived from a single clone and recent studies have demonstrated that tumor cells with tumorigenic potential are limited to a small population of cells, called CICs.<sup>7–14</sup> To cure cancers, it is necessary to control CICs. ALDH, mainly the ALDH1 isoform, oxidizes retinol to retinoic acid in early stages of stem cell differentiation, and hematopoietic and neural stem cells show high ALDH activity.<sup>16,21</sup> ALDH1 is also a cytosolic enzyme responsible for oxidizing a range of aldehydes to their corresponding carboxylic acids, and is involved in the degradation of toxins and anticancer agents.<sup>20</sup> CICs of human multiple myeloma, acute myeloid leukemia, and cancers of brain, lung, and breast also show high ALDH activity.<sup>15,17–19</sup> Therefore, we examined the effect of NEAPP on CICs using ALDH high-expression cells as a marker of CICs. NEAPP treatment decreased cell viability of ALDH-high cells in a comparable level to ALDH-low cells. These results indicated that NEAPP affected not only non-CICs but also CICs. This was consistent with the recent study that the treatment of NEAPP-associated medium had antitumor potential in chemo-resistant ovarian

cancer cells which were supposed to contain abundant CICs, in addition to ordinary ovarian cancer cells.<sup>2,3</sup> Although the ratio of ALDH-high population was reduced more compared to that of ALDH-low population after treatment with NEAPP in an Aldefluor assay, the reduction ratio of ALDH-high cells was comparable to that of ALDH-low cells in a cell viability assay. The discrepancy might be caused by the difference of the assessment procedure that cell viability assay measured the proliferative potential of the cells with NEAPP treatment, whereas the Aldefluor assay measured ALDH activity of cells that survived under NEAPP treatment. Further studies such as *in vivo* research using mouse and elucidation of the mechanism are needed in order to use as an application for medicine.

## V. CONCLUSION

NEAPP induced cell apoptosis in HEC-1 and GCIY cells and showed an antitumor effect in both ALDH-high and -low cells. These results suggested that NEAPP affected not only non-CICs but also CICs.

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## REFERENCES

1. Tanaka H, Mizuno M, Ishikawa K, Nakamura K, Kajiyama H, Kano H, Kikkawa F, Hori M. Plasma-activated medium selectively kills glioblastoma brain tumor cells by down-regulating a survival signaling molecule, AKT kinase. *Plasma Med.* 2011;1:265–277.
2. Iseki S, Nakamura K, Hayashi M, Tanaka H, Kondo H, Kajiyama H, Kano H, Kikkawa F, Hori M. Selective killing of ovarian cancer cells through induction of apoptosis by nonequilibrium atmospheric pressure plasma. *Appl Phys Lett.* 2012;100,113702.
3. Utsumi F, Kajiyama H, Nakamura K, Tanaka H, Mizuno M, Ishikawa K, Kondo H, Kano H, Hori M, Kikkawa F. Effect of indirect nonequilibrium atmospheric pressure plasma on anti-proliferative activity against chronic chemo-resistant ovarian cancer cells in vitro and in vivo. *PLoS One.* 2013;8:e81576.
4. Kalghatgi S, Kelly CM, Cerchar E, Torabi B, Alekseev O, Fridman A, Friedman G, Azizkhan-Clifford J. Effects of nonthermal plasma on mammalian cells. *PLoS One.* 2011;21;6:e16270.
5. Kim CH, Bahn JH, Lee SH, Kim GY, Jun SI, Lee K, Baek SJ. Induction of cell growth arrest by atmospheric non-thermal plasma in colorectal cancer cells. *J Biotechnol.* 2010;150:530–38.
6. Kalghatgi S, Kelly C, Cerchar E, Azizkhan-Clifford J. Selectivity of non-thermal atmospheric-pressure microsecond-pulsed dielectric barrier discharge plasma induced apoptosis in tumor cells over healthy cells. *Plasma Med.* 2011;1:249–63.



7. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med.* 1997;3:730–37.
8. Lessard J, Sauvageau G. Bmi-1 determined the proliferative capacity of normal and leukaemic stem cells. *Nature.* 2003;423:255–260.
9. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci U S A.* 2003;100:3983–8.
10. Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T, Henkelman RM, Cusimano MD, Dirks PB. Identification of human brain tumor initiating cells. *Nature.* 2004;432:396–401.
11. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. *Nature.* 2001;414:105–111.
12. O'Brien CA, Pollett A, Gallinger S, Dick JE. A human colon cancer cell capable of initiating tumor growth in immunodeficient mice. *Nature.* 2007;445:106–10.
13. Ricci-Vitiani L, Lombardi DG, Pilozzi E, Biffoni M, Todaro M, Peschle C, De Maria R. Identification and expansion of human colon-cancer-initiating cells. *Nature.* 2007;445:111–5.
14. Kondo T, Setoguchi T, Taga T. Persistence of a small subpopulation of cancer stem-like cells in the C6 glioma cell line. *Proc Natl Acad Sci U S A.* 2004;101:781–6.
15. Ginestier C, Hur MH, Charafe-Jauffret E, Monville F, Dutcher J, Brown M, Jacquemier J, Viens P, Kleer CG, Liu S, Schott A, Hayes D, Birnbaum D, Wicha MS, Dontu G. ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. *Cell Stem Cell.* 2007;1:555–67.
16. Chute JP, Muramoto GG, Whitesides J, Colvin M, Safi R, Chao NJ, McDonnell DP. Inhibition of aldehyde dehydrogenase and retinoid signaling induces the expansion of human hematopoietic stem cells. *Proc Natl Acad Sci U S A.* 2006;103:11707–12.
17. Jiang F, Qiu Q, Khanna A, Todd NW, Deepak J, Xing L, Wang H, Liu Z, Su Y, Stass SA, Katz RL. Aldehyde dehydrogenase 1 is a tumor stem cell-associated marker in lung cancer. *Mol Cancer Res.* 2009;7:330–8.
18. Matsui W, Wang Q, Barber JP, Brennan S, Smith BD, Borrello I, McNiece I, Lin L, Ambinder RF, Peacock C, Watkins DN, Huff CA, Jones RJ. Clonogenic multiple myeloma progenitors, stem cell properties, and drug resistance. *Cancer Res.* 2008;68:190–7.
19. Pearce DJ, Taussig D, Simpson C, Allen K, Rohatiner AZ, Lister TA, Bonnet D. Characterization of cells with a high aldehyde dehydrogenase activity from cord blood and acute myeloid leukemia samples. *Stem Cells.* 2005;23:752–60.
20. Vasilou V, Pappa A, Petersen DR. Role of aldehyde dehydrogenases in endogenous and xenobiotic metabolism, *Chem Biol Interact.* 2000;129:1–19.
21. Ma I, Allan AL. The role of human aldehyde dehydrogenase in normal and cancer stem cells. *Stem Cell Rev.* 2011;7:292–306.
22. Rahadiani N, Ikeda H, Mamat S, Matsuzaki S, Ueda Y, Umehara R, Tian T, Wang Y, Enomoto T, Kimura T, Aozasa K, Morii E. Expression of aldehyde dehydrogenase 1 (ALDH1) in endometrioid adenocarcinoma and its clinical implications. *Cancer Sci.* 2011;102:903–8.
23. Sakakita H, Ikehara Y. Irradiation experiment on a mouse using a mild-plasma generator for medical applications. *Plasma Fusion Res.* 2010;5:S2117.
24. Ikehara Y, Sakakita H, Shimizu N, Ikehara S, Nakanishi H. Formation of membrane-like structures in clotted blood by mild-plasma treatment during hemostasis. *J Photopolym Sci Technol.* 2013;26:555–7.