Effectiveness of plasma treatment on gastric cancer cells

Koji Torii · Suguru Yamada · Kae Nakamura · Hiromasa Tanaka · Hiroaki Kajiyama · Kuniaki Tanahashi · Naoki Iwata · Mitsuro Kanda · Daisuke Kobayashi · Chie Tanaka · Tsutomu Fujii · Goro Nakayama · Masahiko Koike · Hiroyuki Sugimoto · Shuji Nomoto · Atsushi Natsume · Michitaka Fujiwara · Masaaki Mizuno · Masaru Hori · Hideyuki Saya · Yasuhiro Kodera

Received: 3 March 2014 / Accepted: 11 June 2014 / Published online: 6 July 2014
© The International Gastric Cancer Association and The Japanese Gastric Cancer Association 2014

Abstract

Background Treatment of peritoneal carcinomatosis arising from gastric cancer remains a considerable challenge. In recent years, the anticancer effect of nonequilibrium atmospheric pressure plasma (NEAPP) has been reported in several cancer cell lines. Use of NEAPP may develop into a new class of anticancer therapy that augments surgery, chemotherapy, and radiotherapy.

Method Gastric cancer cells were assessed for changes in cell morphology and rate of proliferation after treatment with NEAPP-exposed medium (PAM). To explore the functional mechanism, caspase 3/7, annexin V, and uptake of reactive oxygen species (ROS) were evaluated, along with the effect of the ROS scavenger *N*-acetylcysteine (NAC).

Results PAM treatment for 24 h affected cell morphology, suggestive of induction of apoptosis. PAM cytotoxicity was influenced by the time of exposure to PAM, the type of cell line, and the number of cells seeded. Cells

treated with PAM for 2 h demonstrated activated caspase 3/7 and an increased proportion of annexin V-positive cells compared with untreated cells. Additionally, ROS uptake was observed in PAM-treated cells, whereas NAC reduced the cytotoxicity induced by PAM presumably through reduction of ROS uptake. Furthermore, CD44 variant 9, which reportedly leads to glutathione synthesis and suppresses stress signaling of ROS, was overexpressed in PAM-resistant cells.

Conclusions PAM treatment induced apoptosis of gastric cancer cells through generation and uptake of ROS. Local administration of PAM could develop into an option to treat peritoneal carcinomatosis.

Keywords Nonthermal atmospheric pressure plasma · Gastric cancer · Apoptosis

K. Torii · S. Yamada · N. Iwata · M. Kanda · D. Kobayashi · C. Tanaka · T. Fujii · G. Nakayama · M. Koike · H. Sugimoto · S. Nomoto · M. Fujiwara · Y. Kodera (✉)
Department of Gastroenterological Surgery (Surgery II), Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, Aichi 466-8550, Japan
e-mail: ykodera@med.nagoya-u.ac.jp

K. Nakamura · H. Kajiyama
Department of Obstetrics and Gynecology, Nagoya University Graduate School of Medicine, Tsurumai-cho 65, Showa-ku, Nagoya 466-8550, Japan

H. Tanaka · M. Mizuno
Center for Advanced Medicine and Clinical Research, Nagoya University Graduate School of Medicine, Tsurumai-cho 65, Showa-ku, Nagoya 466-8550, Japan

H. Tanaka · M. Hori
Plasma Nanotechnology Research Center, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8603, Japan

K. Tanahashi · A. Natsume
Department of Neurosurgery, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan

H. Saya
Division of Gene Regulation, Institute for Advanced Medical Research, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan

Introduction

Gastric cancer accounts for more than 10 % of cancer-related deaths worldwide and remains one of the leading causes of cancer mortality [1]. The commonest pattern of disease failure among patients who underwent curative D2 dissection in the Far East is peritoneal carcinomatosis [2, 3]. Attempts to improve the outcome of patients who have this morbid condition remain a significant challenge, and the development of novel therapeutic modalities and approaches is urgently required. Intraperitoneal administration of taxanes has been explored as a promising strategy [4].

Plasma is essentially an ionized gas consisting of a fraction of ionized atoms or molecules [5], and is sustained by an energy supply containing charged particles such as positively charged ions, electrons, free radicals, excited molecules, and energetic photons. Importantly, it may be a potential alternative to conventional cytotoxic agents or radiotherapy [6]. Cold or nonthermal plasma has been actively deployed for practical purposes [7], and nonequilibrium atmospheric pressure plasma (NEAPP) therapy is currently hoped to fulfill new roles in the field of medical science [8–10]. In recent years, several therapeutic trials have been completed in the fields of tissue sterilization, blood coagulation, wound-healing promotion, and dental bleaching [11–13]. Additionally, it has been reported that plasma can exert antiproliferative effects on various cancer cell lines, possibly through induction of apoptosis [6, 14, 15]. Ultimately, it could develop into a treatment option alongside conventional cytotoxic agents or radiotherapy in the field of oncology [6].

Although the anticancer effect of NEAPP could also apply to gastric cancer, direct exposure of tumors located in the gastric wall to NEAPP treatment may result in adverse effects; it is uncertain what effects ultraviolet or other electromagnetic radiation may have on the surrounding noncancerous tissue. In the current study, the role of a novel form of plasma treatment, exposure to plasma-activated medium (PAM), was explored using gastric cancer cell lines. We evaluated the antiproliferative effect of PAM along with the underlying mechanisms. To our knowledge, this study is the first to explore the therapeutic potential in gastric cancer of NEAPP, which, if delivered intraperitoneally in the form of PAM either alone or in combination with anticancer agents [4], could provide a novel option in the treatment of peritoneal carcinomatosis.

Materials and methods

Production of NEAPP-activated medium

PAM was prepared as reported previously [16]. In brief, 6 mL of RPMI 1640 medium (Life Technologies,

Carlsbad, CA, USA) in a 60-mm dish was exposed to NEAPP, which was generated by the plasma source originally set up at the Plasma Nanotechnology Research Center, Nagoya University Graduate School of Engineering [17]. The flow rate of argon gas and the distance between the plasma source and the sample to be treated are critical parameters for plasma treatment. Although a high flow rate of argon needs to be maintained, it should be optimally controlled lest the medium evaporate. A flow rate of two standard liters per minute and a distance between the plasma source and the medium of 9 mm were established as the tentative standard in the current experimental setup. The duration of exposure of the medium to the plasma was fixed at 5 min. Under this condition, the differences in pH and temperature of the medium before and after plasma treatment were found to be negligible [17].

Cell culture

Human gastric cancer cell lines NUGC4, SC-2-NU, MKN28, and MKN45 and human fibroblast cell line WI-38 were used. NUGC-4 and SC-2-NU were established and maintained at the Department of Gastroenterological Surgery, Nagoya University Graduate School of Medicine. MKN28 and MKN45 were obtained from RIKEN Cell Bank (Tsukuba, Japan). WI-38 was purchased from American Type Culture Collection (Rockville, MD, USA). These cell lines were grown in RPMI 1640 medium supplemented with 10 % fetal bovine serum (Life Technologies), 2 mM L-glutamine, and penicillin (100 UI/mL)–streptomycin (100 UI/mL). All cells were maintained in a humidified incubator at 37 °C with 5 % CO₂.

Cell proliferation assay

Cells were seeded in a 96-well plate containing 100 µL medium in each well at 1×10^3 , 5×10^3 and 1×10^4 cells per well and were incubated for 24 h. The medium was then replaced with 100 µL of freshly prepared PAM. Twenty-four hours later, cell viability was assayed using Premix WST-1 (Takara-Bio, Tokyo, Japan), and the absorbance was measured at 440 nm with a microplate reader. The absorbance values were averaged over three independent experiments.

To evaluate the cytotoxic mechanism of PAM, cell proliferation assay was performed with *N*-acetylcysteine (NAC; Sigma-Aldrich, St Louis, MO, USA), a reactive oxygen species (ROS) scavenger. Cells were seeded in a 96-well plate at 1×10^3 , 5×10^3 , and 1×10^4 cells per well and were incubated for 24 h. The medium was replaced with 100 µL PAM and 4 mM NAC. Twenty-four hours later, cell viability was assayed using WST-1.

Cell apoptosis assay

Cells were seeded in 200 μ L medium in an eight-well culture slide for 24 h, and then the medium was replaced with 200 μ L PAM. Two hours after treatment with PAM, 5 μ M CellEvent caspase 3/7 green detection reagent (Life Technologies) was added, and the cells were incubated for 2 h at 37 °C. The cells were observed using a BZ9000 microscope (Keyence, Osaka, Japan).

Flow-cytometric analysis

A total of 0.5 μ L of diluted (ten times) anti-human CD44 variant 9 (v9) rat IgG antibody (LinkGenomics, Tokyo, Japan) was added to 1×10^6 cells in 50 μ L phosphate-buffered saline. After incubation at 4 °C for 30 min, the cells were stained with secondary anti-rat IgG (H+L) antibody with Alexa Fluor 488 conjugate (Cell Signaling Technology, Danvers, MA, USA). Flow cytometry was performed with a FACSCalibur instrument (BD Biosciences, Franklin Lakes, NJ, USA) to measure the expression of CD44v9.

Cells were seeded at a density of 8×10^5 cells in a 60-mm dish and were incubated for 24 h, and then the medium was replaced with 5 mL PAM. After 2 h treatment with PAM, the cells were harvested and stained with annexin V and propidium iodide (PI), and then flow-cytometric analysis was performed with a FACSCalibur instrument to examine apoptosis.

Detection of intracellular ROS accumulation

Cells were seeded in 200 μ L medium in an eight-well culture slide for 24 h, and then the medium was replaced with 200 μ L PAM. Two hours after PAM treatment, the PAM was replaced with 10 μ M 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA; Life Technologies) diluted in phosphate-buffered saline, and the cells were incubated for 1 h at 37 °C. The cells were observed using a Keyence BZ9000 microscope.

CD44v9 messenger RNA expression in clinical samples

A total of 60 consecutive patients who underwent surgery for gastric cancer from November 2001 to January 2005 at Nagoya University Hospital were examined. The mean age was 63.3 years (range 45–83 years). This study was approved by the Ethics Committee of the hospital, and signed informed consent was obtained from all patients.

Total RNA from human gastric tissues was isolated using ISOGEN (NIPPON GENE, Tokyo, Japan) according to the manufacturer's protocol. Real-time quantitative PCR

analysis was performed as described previously [18]. The PCR primers of CD44v9 were as follows: 5-GA-AGGTGACAGAGCCTCTGGAT-3 (forward) and 5-CATTCCCGTTGGATGACACA-3 (reverse); these amplify a 89-bp product. Each assay was repeated at least three times.

Statistical analysis

Differences between two groups were evaluated using a two-tailed paired Student *t* test. Data are expressed as the mean $\pm$ SD. The presence of a statistically significant difference was denoted by $P < 0.05$. Data were analyzed using JMP version 10 (SAS Institute, Cary, NC, USA).

Results

Cell cytotoxicity of plasma in gastric cancer cell lines

Four gastric cancer cell lines, SC-2-NU, NUGC4, MKN28, and MKN45, were treated with PAM for 24 h to evaluate sensitivity to the plasma. The same four gastric cancer cell lines were plated at 1×10^3 , 5×10^3 , and 1×10^4 cells per well, and culture medium was replaced with PAM after 24 h of incubation. In all cell lines, proliferation was suppressed in a time-dependent manner (Fig. 1a). The sensitivity of PAM differed among the cell lines in that the more highly sensitive cell lines (SC-2-NU and MKN28) were affected by PAM in a relatively shorter exposure time (within 2 min) (Fig. 1a). As for the efficacy according to the cell number, cancer cells seeded at 1×10^4 cells per well were resistant to the PAM generated with an exposure time of 2 min or less. However, PAM generated with an exposure time of more than 2 min overcame the resistance, and showed similar efficacy regardless of the number of cells (Fig. 1a). To evaluate the difference between tumor cells and normal tissue, cytotoxicity was examined in a gastric cancer cell line sensitive to PAM treatment (SC-2-NU), a gastric cancer cell line that is relatively resistant to PAM treatment (MKN45), and a fibroblast cell line representative of normal tissue (WI-38), each at 3×10^3 cells per well. WI-38 was more tolerant of PAM compared with either of the gastric cancer cells, although both cancer and fibroblast cell lines were vulnerable to PAM which was generated with an exposure time longer than 3 min (Fig. 1b).

Plasma induces apoptosis of gastric cancer cells

Morphological changes of the same four gastric cancer cell lines were assessed after exposure to PAM. Cell deformity was noticed after 4 h of exposure, and cell shrinkage and

Fig. 1 a Cell proliferation assay of plasma-activated medium (PAM)-treated cells. A total of 1×10^3 , 5×10^3 , and 1×10^4 cells were seeded into a 96-well plate, and the following day the medium was replaced with PAM. The cell proliferation rate was measured

24 h after medium replacement by the WST-1 assay. **b** Comparison of PAM sensitivity between gastric cancer cells (SC-2-NU and MKN 45) and fibroblast cells (WI-38)

blebbing indicative of apoptosis were observed after 24 h (Fig. 2).

To understand better the mechanism of cell death caused by plasma treatment, the expression of caspase 3/7 as an apoptotic indicator was examined. Most cells that were shrunk with rounded morphology after PAM treatment were shown to overexpress caspase 3/7 (Fig. 3).

To investigate further the induction of apoptosis, flow-cytometric analysis was performed. A greater proportion of cells that underwent PAM treatment were distributed in an annexin V-positive and PI-negative region when compared with untreated cells in three of the four cell lines, suggesting that these cells had undergone apoptosis but not necrosis (Fig. 4).

ROS uptake in plasma-treated gastric cancer cells

To explore the underlying mechanism of apoptosis, ROS uptake into cells was measured. A total of 1×10^4 cells

were seeded, and after 2 h of PAM treatment, the PAM was replaced with CM-H₂DCFDA and ROS uptake was examined. Frequent ROS uptake was seen in apoptotic cells with concomitant morphological changes, whereas no ROS uptake was found in untreated cells (Fig. 5).

To confirm the ROS effect of PAM, cell proliferation assays were performed using PAM containing NAC as a ROS scavenger. All cell lines showed resistance to PAM, although a cytotoxic effect became apparent after 5 min of exposure to PAM in three of the four cell lines (Fig. 6).

Since correlation of CD44v9 expression with ROS resistance has been reported [18], CD44v9 expression was evaluated by fluorescence-activated cell sorting (Fig. 7). As expected, cell lines that were more sensitive to PAM (SC-2-NU and MKN28) exhibited low CD44v9 expression, whereas less sensitive cell lines (NUGC4 and MKN45) exhibited fairly high CD44v9 expression. Additionally, measurement of CD44v9 expression in clinical samples was conducted by real-time quantitative PCR. The 5-year

Fig. 2 Morphological changes of gastric cancer cell lines after treatment with PAM. A total of 1×10^5 cells were seeded into a 12-well plate, and the following day the medium was replaced with PAM. Cells became more rounded after 4 h, and were smaller and spherical after 24 h. Round and prominent cytoplasm were also observed (arrowheads)

Fig. 3 Caspase 3/7 activation by PAM treatment. A total of 1×10^4 cells were cultured on slides, and medium was replaced with PAM. Caspase 3/7 activity in the cell nuclei was measured by fluorescence microscopy. Caspase 3/7 expression was found in the nuclei of cells that exhibited morphological changes. The scale bar corresponds to 100 μm

Fig. 4 Apoptotic changes induced by PAM treatment. A total of 8×10^5 cells were seeded, and were harvested after 2 h of incubation with PAM. Cells were stained with annexin V and propidium iodide (PI), and were measured by fluorescence-activated cell sorting.

Annexin V-positive and PI-negative ratios were increased in PAM-treated cells; only SC-2-NU cells showed an inverse result. This may be a result of poor affinity for annexin V

Fig. 5 Measurement of reactive oxygen species (ROS) uptake resulting from PAM treatment. A total of 1×10^4 cells were seeded, and then PAM was replaced with 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA), and ROS uptake was measured. Increased ROS uptake was found in apoptotic cells with morphological changes compared with untreated cells. The scale bar corresponds to 100 μ m

Fig. 6 Cell proliferation assay following PAM treatment and ROS scavenger treatment. A total of 1×10^3 , 5×10^3 , and 1×10^4 cells were seeded, and the following day medium was replaced with PAM

containing *N*-acetylcysteine (NAC). Cell proliferation assays revealed that all cell lines showed resistance to PAM; however, the greatest effect was observed with PAM exposure for 5 min

survival rate of the high CD44v9 expression group was 64.5 %, whereas that of the low CD44v9 expression group was 47.2 % ($P = 0.14$). Eight of 60 patients had gastric cancer with pathologically confirmed peritoneal dissemination, and the patients with peritoneal dissemination tended to have lower CD44v9 expression than those without peritoneal dissemination ($P = 0.123$, data not shown).

Discussion

The cytotoxicity of NEAPP observed with various cancer cell lines indicated its potential as a promising treatment option in the field of oncology [17, 20, 21]. However, a device to directly irradiate lesions deep within the body with NEAPP is currently unavailable. In addition, the potential for adverse effects from ultraviolet rays and other electromagnetic waves through direct exposure to NEAPP will also have to be considered. To resolve these issues, we

explored herein the feasibility of treatment with PAM since, in our earlier attempts, PAM was found to have a cytotoxic effect on glioma cells while circumventing exposure to ultraviolet or other electromagnetic wavelengths as in the case of direct irradiation with NEAPP [22]. Intraperitoneal administration of cytotoxic agents has been found to be effective in the treatment of peritoneal surface malignancy, including metastasis from gastric cancer [4, 23]. However, novel agents designed for intraperitoneal administration remain scarce [24]. As a liquid agent with a cytotoxic effect specific to cancer, PAM either alone or with cytotoxic agents could be a breakthrough in the treatment of gastric cancer with peritoneal dissemination.

Ma et al. [25] reported that plasma generates several types of ROS, and this could at least partially account for the cytotoxicity induced by plasma treatment, although details of the sequence of events that occur after the exposure to ROS and the relevance of other possible

Fig. 7 CD44 variant 9 (*CD44v9*) expression of gastric cancer cell lines. CD44v9 expression was examined by fluorescence-activated cell sorting, and the CD44v9-positive ratio in gastric cancer cell lines is shown. An inverse correlation between CD44v9 expression and PAM sensitivity was found

mechanisms that lead to cytotoxicity remain unclear. Our results support this and other hypotheses that attributed the cytotoxic effects of plasma to apoptosis resulting from ROS uptake into tumor cells [14, 26]. Only SC-2-NU cells showed an inverse result. We thought this may be dependent on poor affinity for annexin V, because similar morphological changes and caspase 3/7 activation were found, and the proportion of PI-positive cells was increased in PAM-treated cells compared with untreated cells. Furthermore, sensitivity to PAM differed among the four gastric cancer cell lines tested in the current study. In a study using prostate cancer cell lines, Chan et al. [27] reported that the variation among cell lines in the amount of ROS accumulated when triggered by the same exogenous oxidative stimuli resulted in differing levels of cytotoxicity. Further investigations to elucidate the mechanisms behind the sensitivity to plasma treatment are warranted, and CD44 could be a candidate molecule for scrutiny.

CD44 is essentially a major component of extracellular matrices and has some variant isoforms, which arise by the alternative splicing of variant exons. One of these variants, CD44v9, is considered as a candidate stem cell marker for gastric cancer [28], and is significantly associated with recurrence, mortality, and resistance to chemotherapy or radiotherapy [29–31] through its role as a common downstream effector of RAS [32]. More recently, the correlation between CD44v9 expression and resistance to ROS was reported [19]. In the current study, cells with low sensitivity to PAM (NUGC4 and MKN45) overexpressed CD44v9, whereas cells with high sensitivity to PAM (SC-2-NU and MKN28) did not express CD44v9. These

findings are compatible with the implicated role of ROS in the cytotoxic effect of NEAPP and account for the mechanism that confers cancer cells with resistance to NEAPP treatment. Cells expressing CD44v9 reportedly keep xCT (a subunit of glutamate-cystine exchange transporter) activated and cysteine uptake promoted, leading to intracellular synthesis of glutathione, which plays a key role in neutralization of free radicals and prevention of such stress signaling inducing p38 mitogen-activated protein kinase activation which could lead to cell-cycle arrest and senescence [19]. Additionally, Yoshida et al. [33] reported that the epithelial splicing regulatory protein 1–CD44 variant–xCT–glutathione axis renders numerous epithelial cancer cells resistant to ROS. In the current study, gastric cancer patients with peritoneal dissemination tended to show lower CD44v9 expression in cancer tissues. This implies further that PAM could be suitable for treatment of peritoneal carcinomatosis.

Tanaka et al. [22] revealed that the cytotoxicity of PAM was specific to malignant cells. Nicco et al. [34] showed that normal cells were more tolerant of exogenous oxidative stress than cancer cells, which supports the differential cytotoxicity of PAM between normal and malignant cells. Our results also show that WI-38 fibroblast cells are more tolerant of PAM treatment than gastric cancer cells, although even WI-38 cells die as a result of prolonged exposure to PAM. Thus, PAM treatment is a treatment option with a therapeutic window, and stringent conditions may need to be observed for safety and efficacy.

In conclusion, PAM has a significant cytotoxic effect on gastric cancer cells by an indirect exposure method, and apoptosis due to ROS uptake into tumor cells might be the underlying mechanism, and the expression of CD44v9 may be associated with resistance. Furthermore, PAM treatment could selectively target cancer cells under optimal conditions. Therefore, local administration of PAM could be a promising therapeutic option for gastric cancer patients with peritoneal dissemination.

References

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin*. 2011;61:69–90.
2. Nakamura K, Ueyama T, Yao T, Xuan ZX, Ambe K, Adachi Y. Pathology and prognosis of gastric carcinoma. Findings in 10,000 patients who underwent primary gastrectomy. *Cancer*. 1992;70:1030–7.
3. Yu CC, Levison DA, Dunn JA, Ward LC, Demonakou M, Allum WH, et al. Pathological prognostic factors in the second British Stomach Cancer Group trial of adjuvant therapy in resectable gastric cancer. *Br J Cancer*. 1995;71:1106–10.
4. Ishigami H, Kitayama J, Kaisaki S, Hidemura A, Kato M, Otani K, et al. Phase II study of weekly intravenous and intraperitoneal

- paclitaxel combined with S-1 for advanced gastric cancer with peritoneal metastasis. *Ann Oncol.* 2010;21(1):67–70.
5. Fridman A. *Plasma chemistry*. Cambridge: Cambridge University Press; 2008.
 6. Vandamme M, Robert E, Pesnel S, Barbosa E, Dozias S, Sobilo J, et al. Antitumor effect of plasma treatment on U87 glioma xenografts: preliminary results. *Plasma Process Polym.* 2010;7:264–73.
 7. Yamazaki H, Ohshima T, Tsubota Y, Yamaguchi H, Jayawardena JA, Nishimura Y. Microbicidal activities of low frequency atmospheric pressure plasma jets on oral pathogens. *Dent Mater J.* 2011;30:384–91.
 8. Fridman G, Friedman G, Gutsol A, Shekhter AB, Vasilets VN, Fridman A. Applied plasma medicine. *Plasma Process Polym.* 2008;5:503–33.
 9. Morfill GE, Kong MG, Zimmermann JL. Focus on plasma medicine. *New J Phys.* 2009;11(11):115011.
 10. Yousfi M, Merbahi N, Pathak A, Eichwald O. Low-temperature plasmas at atmospheric pressure: toward new pharmaceutical treatments in medicine. *Fundam Clin Pharmacol.* 2013. doi:10.1111/fcp.12018.
 11. Brun P, Brun P, Vono M, Venier P, Tarricone E, Deligianni V, et al. Disinfection of ocular cells and tissues by atmospheric-pressure cold plasma. *PLoS ONE.* 2012;7(3):e33245.
 12. Fridman G, Peddinghaus M, Balasubramanian M, Ayan H, Fridman A, Gutsol A, et al. Blood coagulation and living tissue sterilization by floating-electrode dielectric barrier discharge in air. *Plasma Chem Plasma Process.* 2006;26:425–42.
 13. Lee JK, Kim, Byun JH, Kim KT, Kim GC, Park GY. Biochemical applications of low temperature atmospheric pressure plasmas to cancerous cell treatment and tooth breaching. *Jpn J Appl Phys.* 2011;50:08JF01.
 14. Sato T, Yokoyama M, Johkura K. A key inactivation factor of HeLa cell viability by a plasma flow. *J Phys D Appl Phys.* 2011;44(37):372001.
 15. Sensenig R, Kalghatgi S, Cerchar E, Fridman G, Shereshevsky A, Torabi B, et al. Non-thermal plasma induces apoptosis in melanoma cells via production of intracellular reactive oxygen species. *Ann Biomed Eng.* 2011;39:674–87. Retraction in: *Ann Biomed Eng.* 2013;41(3):656.
 16. Utsumi F, Kajiyama H, Nakamura K, Tanaka H, Mizuno M, Ishikawa K, et al. 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(12):e81576.
 17. Iseki S, Nakamura K, Hayashi M, Tanaka H, Kondo H, Kajiyama H, et al. Selective killing of ovarian cancer cells through induction of apoptosis by nonequilibrium atmospheric pressure plasma. *Appl Phys Lett.* 2012;100(11):113702.
 18. Ito T, Yamada S, Tanaka C, Ito S, Murai T, Kobayashi D, et al. Overexpression of L1CAM is associated with tumor progression and prognosis via ERK signaling in gastric cancer. *Ann Surg Oncol.* 2014;21(2):560–8.
 19. Ishimoto T, Nagano O, Yae T, Tamada M, Motohara T, Oshima H, et al. CD44 variant regulates redox status in cancer cells by stabilizing the xCT subunit of system xc⁻ and thereby promotes tumor growth. *Cancer Cell.* 2011;19:387–400.
 20. Kim CH, Kwon S, Bahn JH, Lee K, Jun SI, Rack PD, et al. Effects of atmospheric nonthermal plasma on invasion of colorectal cancer cells. *Appl Phys Lett.* 2010;96(24):243701.
 21. Fridman G, Shereshevsky A, Jost M, Brooks A, Fridman A, Gutsol A, et al. Floating electrode dielectric barrier discharge plasma in air promoting apoptotic behavior in melanoma skin cancer cell lines. *Plasma Chem Plasma Process.* 2007;27:163–76.
 22. Tanaka H, Mizuno M, Ishikawa K, Nakamura K, Kajiyama H, Kano H, et al. Plasma-activated medium selectively kills glioblastoma brain tumor cells by down-regulating a survival signaling molecule, AKT kinase. *Plasma Med.* 2011;1(3–4):265–77.
 23. Armstrong DK, Bundy B, Wenzel L, Huang HQ, Baergen R, Lele S, et al. Intraperitoneal cisplatin and paclitaxel in ovarian cancer. *N Engl J Med.* 2006;354:34–43.
 24. Heiss MM, Murawa P, Koralewski P, Kutarsa E, Kolesnik OO, Ivanchenko VV, et al. The trifunctional antibody catumaxomab for the treatment of malignant ascites due to epithelial cancer: results of a prospective randomized phase II/III trial. *Int J Cancer.* 2010;127:2209–21.
 25. Ma R, Feng H, Li F, Liang Y, Zhang Q, Zhu W, et al. An evaluation of anti-oxidative protection for cells against atmospheric pressure cold plasma treatment. *Appl Phys Lett.* 2012;100(12):123701.
 26. Ninomiya K, Ishijima T, Imamura M, Yamahara T, Enomoto H, Takahashi K, et al. Evaluation of extra- and intracellular OH radical generation, cancer cell injury, and apoptosis induced by a non-thermal atmospheric-pressure plasma jet. *J Phys D Appl Phys.* 2013;46(42):425401.
 27. Chan SW, Nguyen PN, Ayele D, Chevalier S, Aprikian A, Chen JZ. Mitochondrial DNA damage is sensitive to exogenous H₂O₂ but independent of cellular ROS production in prostate cancer cells. *Mutat Res.* 2011;716(1–2):40–50.
 28. Jang BI, Li Y, Graham DY, Cen P. The role of CD44 in the pathogenesis, diagnosis, and therapy of gastric cancer. *Gut Liver.* 2011;5(4):397–405.
 29. Liu J, Ma L, Xu J, Liu C, Zhang J, Liu J, et al. Spheroid body-forming cells in the human gastric cancer cell line MKN-45 possess cancer stem cell properties. *Int J Oncol.* 2013;42(2):453–9.
 30. Mayer B, Jauch KW, Gunthert U, Figdor CG, Schildberg FW, Funke I, et al. De novo expression of CD44 and survival in gastric cancer. *Lancet.* 1993;342(8878):1019–22.
 31. Shibuya Y, Okabayashi T, Oda K, Tanaka N. Ratio of CD44 epithelial variant to CD44 hematopoietic variant is a useful prognostic indicator in gastric and colorectal carcinoma. *Jpn J Clin Oncol.* 1998;28(10):609–14.
 32. Györfy B, Schäfer R. Biomarkers downstream of RAS: a search for robust transcriptional targets. *Curr Cancer Drug Targets.* 2010;10:858–68.
 33. Yoshida GJ, Saya H. Inversed relationship between CD44 variant and c-Myc due to oxidative stress-induced canonical Wnt activation. *Biochem Biophys Res Commun.* 2014;443(2):622–7.
 34. Nicco C, Laurent A, Chereau C, Weill B, Batteux F. Differential modulation of normal and tumor cell proliferation by reactive oxygen species. *Biomed Pharmacother.* 2005;59(4):169–74.