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A Review on the Selective Apoptotic Effect of Nonthermal Atmospheric-Pressure Plasma on Cancer Cells

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ABSTRACT: Nonthermal atmospheric-pressure plasma (NTAPP) is defined as a partially ionized gas containing electrically charged particles. Due to its low temperature and generation in atmospheric pressure, NTAPP has been useful for biomedical applications such as sterilization and wound healing. Recently, several groups have reported that NTAPP is able to induce apoptosis selectively in cancer cells, which opens a new discussion regarding whether NTAPP can be a competitive cancer therapy. Although most research has continued with *in vitro* experiments, a few groups have already demonstrated that NTAPP can be applied to xenograft mouse models to decrease the size of tumors. However, the mechanism of how the NTAPP efficiently induces apoptosis in cancer cells is not well understood. Results of current studies strongly suggest reactive oxygen species (ROS) and reactive nitrogen species (RNS) to be the primary components that induce DNA double-strand breaks (DSBs) to cause apoptosis. Cancer cells are, in general, defective in genes responsible for cell cycle control. Thus, the effect of NTAPP on the signaling and checkpoint pathways to control the cell cycle should be examined to understand the molecular mechanism of the selective apoptosis by NTAPP. This review evaluates the selective effect of NTAPP on cancer cells and their molecular mechanisms; our results support the potential of NTAPP as an efficient anticancer therapy in near future.

KEY WORDS: Nonthermal atmospheric pressure plasma, apoptosis, cancer cells, p53, anticancer therapy

I. GENETIC BACKGROUND OF CANCER

Cancer is regarded as a genetic disease because it is caused by genetic damage in genes that regulate the growth and proliferation of cells. The accumulated mutations of three classes of genes often result in the losses of cellular control in growth and proliferation that give rise to cancer: proto-oncogenes, tumor-suppressor genes, and caretaker genes. These genes normally encode proteins that control a cell proliferation process known as a cell cycle. Proto-oncogenes usually promote the cell cycle for cell duplication, and their function can be explained as an accelerator of the cell cycle. Their mutations transform them into oncogenes that excessively activate proliferation and provide ways to become cancer. Oncogenic mutations usually hyperactivate the function of the gene and thereby the signaling pathway in which it plays a role. Tumor-suppressor genes normally suppress cell cycle and proliferation and are assumed to provide a brake on the

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cell cycle. Their mutations inactivate the genes and lead to unrestrained cell division. Caretaker genes normally function to maintain genomic integrity by repairing and protecting cells from various DNA damage. Caretaker genes also control cell death when genetic damage is unrepairable. Malfunction of these genes makes cells accumulate mutations, including proto-oncogenes and tumor-suppressor genes at an increased rate, which might lead to cancer (for a review, see Deininger[1]).

Apoptosis is a well-known form of programmed cell death that removes damaged and unwanted cells. It serves as a crucial mechanism to defend tissues and organs from various types of stress and damage.[2] Apoptosis is marked by a sequence of morphological changes, including shrinking and fragmentation of cells into a small, membrane-bound, apoptotic body that can be engulfed by neighboring cells. In cells undergoing apoptosis, the nucleus is condensed, DNA is fragmented, and a cascade of proteases known as caspases becomes activated (for a review, see Elmore[2]).

Usually, severe damage by anticancer therapies to induce apoptosis in cancer cells also results in the death of normal cells, causing side effects. Thus, any approach that induces selective apoptosis in cancer cells with the minimal detrimental effect on surrounding normal cells is potentially an ideal treatment for cancer. Many anticancer therapies and agents have been developed, but all current approaches still face significant challenges, including drug resistance, low therapeutic efficiency, and cancer cell selectivity.

II. BIOLOGICAL APPLICATION OF NONTHERMAL ATMOSPHERIC-PRESSURE PLASMA

Plasma is described as a quasineutral mixture of charged particles and radicals in a partially ionized gas. Recently, many studies have attempted to take advantage of the low temperature of nonthermal atmospheric-pressure plasmas (NTAPPs) and the controllability of plasma chemistry and kinetics of NTAPPs for biomedical applications.[3–5] Three main types of device generate NTAPP for biomedical applications: plasma needle, plasma jet, and dielectric barrier discharge (DBD).[5] The gas component and the strength and pulse duration of the electric field of NTAPP determine the exact composition of the plasma. NTAPPs are easily generated in air and can be used without causing thermal damage to cells. The effect of NTAPP on living tissues includes sterilization, cell death, wound healing, and cell migration changes (for reviews, see Kong et al.[4] and Park et al.[6]). The various effects of plasma depend on plasma dosage and the complex chemical composition of the plasma.

Several gas sources have been used for plasma generation (Table 1). Inert gases (Ar and He), nitrogen, and air have been frequently used to generate plasma for biological applications. Recently, researchers have been prone to add oxygen to inert gas to improve its biological effects. Air could also be considered as a combination of nitrogen and oxygen. Kim et al. showed that NTAPP generated using helium mixed with oxygen is much more efficient in blocking the migration and invasion of SW480 cells than NTAPP with helium alone.[7]

TABLE 1. Apoptotic Death of Cancer Cells by the Treatment of Nonthermal Atmospheric-Pressure Plasma *In vitro*

Cell Name	Cell Type	Device Type	Gas	NTAPP Treatment	Reference
NOS2, NOS3	Human epithelial ovarian carcinoma cell	?	Ar	Plasma-activated medium	Utsumi *et al.*, 2013, *PLoS ONE*[16]
SK-HEP-1	Human hepatocellular carcinoma cell	Jet	He	Direct	Gweon *et al.*, 2011, *Applied Physics Letters*[9]
A549	Human pulmonary carcinoma cell	DBD	Ar/O_2	Direct	Jun Huang et al., 2011, *Applied Physics Letters*[47]
HepG2	Human hepatocellular carcinoma cell	Jet	He/O_2	Direct	Yan *et al.*, 2012, *Plasma Process and Polymers*[48]
HepG2	Human hepatocellular carcinoma cell	Jet	He/O_2	Direct	Yan *et al.*, 2010, *IEEE Transactions on Plasma Science*[49]
HeLa	Human cervical carcinoma cell	Jet	N_2 or Air	Direct	Ahn *et al.*, 2011, *PLoS ONE*[50]
A2058	Human melanoma cell line	FE-DBD	?	Direct	Sensenig *et al.*, 2010, *Annals of Biomedical Engineering*[51]
SW480	Human colorectal cancer cell	Jet	He or He/O2	Direct	Kim *et al.*, 2010, *Applied Physics Letters*[7]
HCT116, SW480, LoVo	Human colorectal cancer cell	?	He/O2	Direct	Kim *et al.*, 2010, *Journal of Biotechnology*[52]
B16F10	Mouse melanoma cancer cell	?	Air	Direct	Kim *et al.*, 2010, *Applied Physics Letters*[53]
U87MG, HCT116	Glioblastoma cancer cell, Human colorectal cancer cell	FE-DBD	Air	Direct	Vandamme *et al.*, 2012, *International Journal of Cancer*[14]

Table 1. (Continued)

Mel-RM, Mel-007, Mel-JD	Human melanoma cell	Jet	He	?	Ishaq *et al*., 2014, *Molecular Biology of the Cell*[11]
MSK QLL1, SCC1483, SCC15, SCC25	Human oral cavity cancer cell	?	He/O_2	Direct	Chang *et al*., 2014, *Archives of Biochemistry and Biophysics*[54]

III. SELECTIVE APOPTOSIS OF VARIOUS CANCER CELLS BY NTAPP

In efforts to develop NTAPP as a new cancer therapy, the clinical application of NTAPP for mammalian cells has mainly focused on its effect on apoptotic cell death. Many studies have shown that NTAPP generated by different plasma devices induce apoptotic death in various kinds of mammalian cancer cells (Table 1). However, the apoptotic effect of NTAPP is not enough to support NTAPP as a potential anticancer therapy. To develop NTAPP as a potential cancer therapy, the selective effect of NTAPP on cancer cells over normal cells must be evaluated.

Several research groups have reported the differential effect of NTAPP on cancer cells and normal cells. Volotskova et al. observed that two different murine skin cancer cells demonstrate more increased sensitivity to NTAPP than the wild-type murine keratinocytes.[8] Gweon et al. reported the differential cell death of human liver cancer cells (SK-HEP-1) and normal cells (THLE-2) by NTAPP treatment. They also showed the different detachment cell death of human liver cancer cells (SK-HEP-1) and normal cells (THLE-2) by NTAPP treatment.[9] They also showed the different detachment characteristics of cancer and normal cells in response to the atmospheric pressure plasma. Using an annular-type dielectric barrier discharge (DBD) NTAPP-generating apparatus with an He gas-feeding system, our group also showed a preferential antiproliferative effect of NTAPP on the cancerous HeLa cells over normal primary fibroblast IMR90 cells (Fig. 1A).[10] When we applied the same NTAPP exposure condition for the apoptosis of HeLa cells to various human cancer cells from oral squamous carcinoma, colorectal carcinoma, malignant melanoma, lung cancer, uterine sarcoma, colorectal adenocarcinoma, to hepatocarcinoma (Table 2), we observed highly selective apoptotic cell death in these cancer cells.[10] Meanwhile, Ishaq et al. showed that NTAPP generated with He gas by a jet induced apoptosis much more efficiently in human melanoma cells (Mel-RM, Mel007, Mel-JD) than in normal melanocytes and fetal lung fibroblasts (MRC5) (Fig. 1B).[11] Panngom et al. also reported that human lung cancer cell lines showed a greater increase in cytotoxicity than in normal lung

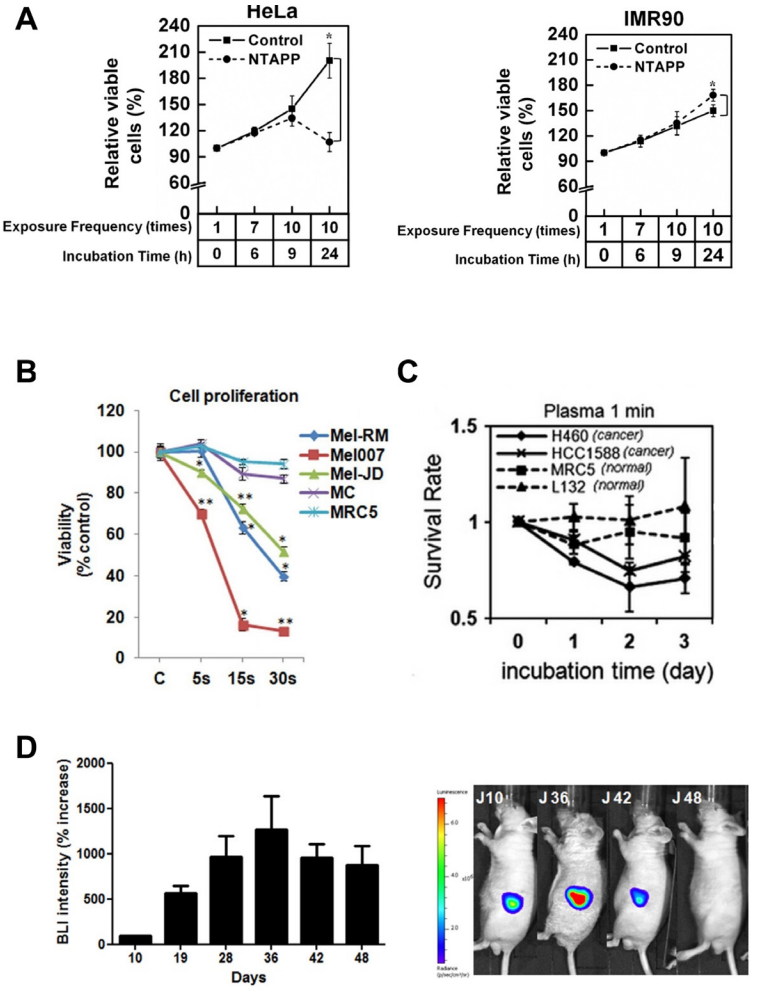

FIG. 1: Selective apoptosis of various cancer cells by nonthermal atmospheric pressure plasma. **(a)** HeLa and IMR90 cells were exposed with 5 V input NTAPP for 30 s every hour 10 times and further incubated for 15 h. Cell viability was evaluated with MTT assays (Ma *et al.*,[10] open access). **(b)** Melanoma (Mel-RM, Mel007, Mel-JD) and melanocytes (MC) were treated with NTAPP for 5–30 s and cell viability was monitored via cell titer nonradioactive cell proliferation assay (Ishaq *et al.,*[11]with the permission of Molec. Biol. Cell.). **(c)** Survival rate of two lung cancer cell lines (H460 and HCC1588) and two lung normal cell lines (MRC5 and L132) was presented after cells were treated with plasma for 1 min and incubated for 3 days.[12] **(d)** Tumor orthotropic xenografts were prepared by injecting MIA PaCa2-luc cells into the tail of pancreas of female Swiss nude mice. Mice were treated with NTAPP for 10 min every 10 days at a repetition rate of 2 kHz. Tumor volumes were monitored by bioluminescence from day 10 to day 48. Values represent means ±SEM, n=9 for each group. Exemplary results from *in vivo* bioluminescence imaging (BLI) showed an increase of BLI intensity from D10 to D36, but a decrease from D42 (Brulle *et al.*,[13] open access).

TABLE 2. Differential Effects of Nonthermal Atmospheric-Pressure Plasma on Cancer and Normal Cells

Cancer Cell Name (Type)	Normal Cell Name (Type)	Effect	Reference
HeLa (Human adenocarcinoma) YD-9 (Human oral squamous carcinoma) G361 (Human malignant melanoma) HCT116 p53+/+ (Human colorectal carcinoma) HCT116-E6 p53-/- (Human colorectal carcinoma) RKO (Human colorectal carcinoma) MES-SA (Human uterine sarcoma) MES-SA/Dx5 (Human uterine sarcoma) HepG2 (Human hepatocellular carcinoma) LoVo (Human colorectal adenocarcinoma) DLD-1 (Human colorectal adenocarcinoma) HCT15 (Human colorectal adenocarcinoma) HCT15/CL02 (Human colorectal adenocarcinoma) HT29 (Human colorectal carcinoma) H1299 (Human non-small cell lung cancer)	IMR90 (Human lung fibroblast)	Selective anticancer effect, especially for p53-mutated cells	Ma *et al*., 2014, *PLoS ONE*[10]
SK-HEP-1 (Human liver cancer cell)	THLE-2 (Normal liver cells)	Cancer cells detached more readily than normal cells	Gweon *et al*., 2011, *Applied Physics Letters*[9]
Mel-RM, Mel007, Mel-JD (human melanoma cell line)	MRC5 (Human fetal lung fibroblast) Melanocyte (Primary human epidermal melanocyte)	Selectively apoptotic effect on cancer cell lines	Ishaq *et al.*, 2014, *Molecular Biology of the Cell*[11]
H460, HCC 1588 (Human lung cancer cell)	MRC5, L132 (Human lung normal cell)	Preferential killing of cancer cells to normal cells	Panngom *et al.*, 2013, *Cell Death and Disease*[12]

cell lines (MRC5 and L132) when a DBD non-thermal plasma based on air was applied (Fig. 1C).[12] The preferential effects of NTAPP on various cancer cells reported in the current literature are summarized in Table 2.

When NTAPP was directly applied to xenograft tumors using murine models with a glioblastoma cancer cell line and a pancreatic carcinoma *in vivo*, Brulle et al. and Vandamme et al. reported that cell proliferation and tumor volume decreased (Fig. 1D).[13,14] Altogether, these results strongly support the feasibility of proper NTAPP exposures for selective apoptosis of cancer cells, suggesting a high potential of NTAPP as a new cancer therapy.

IV. SELECTIVE APOPTOSIS OF CANCER CELLS BY DIRECT OR INDIRECT NTAPP

In the previous section, we discussed the highly selective apoptotic death of cancer cells by NTAPP, when cells were directly exposed to NTAPP. Although most studies have reached the same conclusion that NTAPP can efficiently induce apoptosis of cancer cells, the method of NTAPP treatment has varied. In most research, NTAPP has been directly applied to the cells seeded in the dish with media.

Interestingly, selective apoptosis of cancer cells has also been observed when cells were treated with plasma-activated medium (PAM). Tanaka et al. reported that glioblastoma cells were selectively killed by PAM through apoptosis, while normal human brain astrocytes were not affected.[15] Similarly, Utsumi et al. also reported that the viability of chronic chemoresistant ovarian cancer cells, NOS2 and NOS3, decreased by ~30% after cells were treated with NTAPP-activated medium (NTAPP-AM) (Fig. 2A and B).[16] Surprisingly, they also reported that when a murine subcutaneous tumor model was developed by the inoculation of NOS2, the direct injection of NTAPP-AM to the tumor reduced its size by 66%.[16] A DBD-type NTAPP device with an Ar gas source was used in these experiments. These results suggest that plasma-activated medium has also an antitumor effect on cancer cells *in vitro* and *in vivo*. However, when we only exposed the medium to NTAPP under the same conditions as we treated NTAPP to HeLa cells (Fig. 1A), and then NTAPP-unexposed HeLa cells were incubated with NTAPP-exposed medium, these cells did not show a significant decrease in viability (Fig. 2C). In addition, when HeLa cells in the medium were directly exposed to NTAPP under the same conditions and the medium was replaced with fresh medium after the exposure, the antiproliferative effect of NTAPP on HeLa cells was highly diminished (Fig. 2D). Our experiments demonstrate that the medium exposed to NTAPP plays an important role for the apoptosis of cancer cells and the indirect apoptotic effect of NTAPP is not efficient.

The discrepancy regarding the apoptotic effect of indirect NTAPP (Fig. 2) may have been derived from differences in the intensity of electric and magnetic fields applied to generate plasma, plasma dosage, and the complex chemical compositions of plasma including the reactive oxygen species and charged particles.

V. ANTIPROLIFERATIVE ROLES OF ROS AND RNS GENERATED BY NTAPP

Reactive oxygen species (ROS) are chemically reactive radicals, ions, or molecules containing free oxygen radicals and a byproduct of normal metabolism. Basal levels of ROS activate numerous signaling cascades to promote cell proliferation under normal physiological conditions.[14,17,18] However, excessive ROS levels induce oxidative stress and directly attack DNA, protein, lipids, and other cellular components, ultimately contributing to cell senescence and apoptosis.[19,20] Reactive nitrogen species (RNS) are a family of molecules from the reaction of nitric oxide (·NO) and superoxide ($O_2^{\cdot-}$) and act with ROS to damage cells. Especially peroxynitrite ($ONOO^-$) can cause severe damage to an organism by depleting NO, a key mediator of many vascular functions.[21]

Many plasma research groups have presented evidence suggesting that ROS and RNS are the major players of NTAPP-induced apoptosis *in vitro* and *in vivo* (Table 3). In fact, when intracellular ROS was observed in NTAPP-treated and untreated cells with dichlorofluorescein (DCF), an oxidation-sensitive dye that can detect the intracellular ROS, bright DCF staining was only observed in NTAPP-treated cells (Fig. 3A).[22] This result suggests that exposure to NTAPP increases not only the extracellular but also the intracellular ROS. Our group wanted to determine whether the highly selective antiproliferative effect of NTAPP depends on the intracellular ROS or the extracellular ROS. For this purpose, two well-known thiol antioxidants, N-acetylcysteine (NAC) and sodium pyruvate (SP), were used.[10,23] To examine the role of intracellular ROS in the selective antiproliferative effect of NTAPP, we assessed the relative cell viability of HeLa cells with and without NTAPP treatment in the presence of different concentrations of NAC (3 and 5 mM). HeLa cell viability was sharply decreased by NTAPP exposure, but it was recovered by the presence of NAC in a concentration-dependent manner (Fig. 3B). The antiproliferative effect of extracellular ROS generated by NTAPP was also examined using an extracellular ROS scavenger, SP. When HeLa cells were exposed to NTAPP in the presence of SP, the relative number of viable cells was increased in a concentration-dependent way, compared with NTAPP-exposed cells that were not treated with SP (Fig. 3C). These results demonstrate that both intra- and extracellular ROS play a major role in the antiproliferative effect of NTAPP.

Our group was especially interested in whether NO is easily generated when plasma meets N_2 and O_2 in the air because NO is known to be an important cellular messenger molecule involved in many physiological and signaling pathways in mammals.[24,25] When we detected NO in the media with Griess reagent assays[26] after cells were exposed to NTAPP, the concentration of NO in the media was increased more than 7−8 fold and was persistent for 15 h after NTAPP exposure treatment compared with the untreated control.[10] We then assessed the antiproliferative effect of NO generated in the media by NTAPP with an NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (carboxy-PTIO). There was no difference in viability by NTAPP exposure regardless of the addition of carboxy-PTIO (Fig. 3D), suggesting that the extracellular NO generated by NTAPP does not influence the antiproliferative effect of NTAPP.

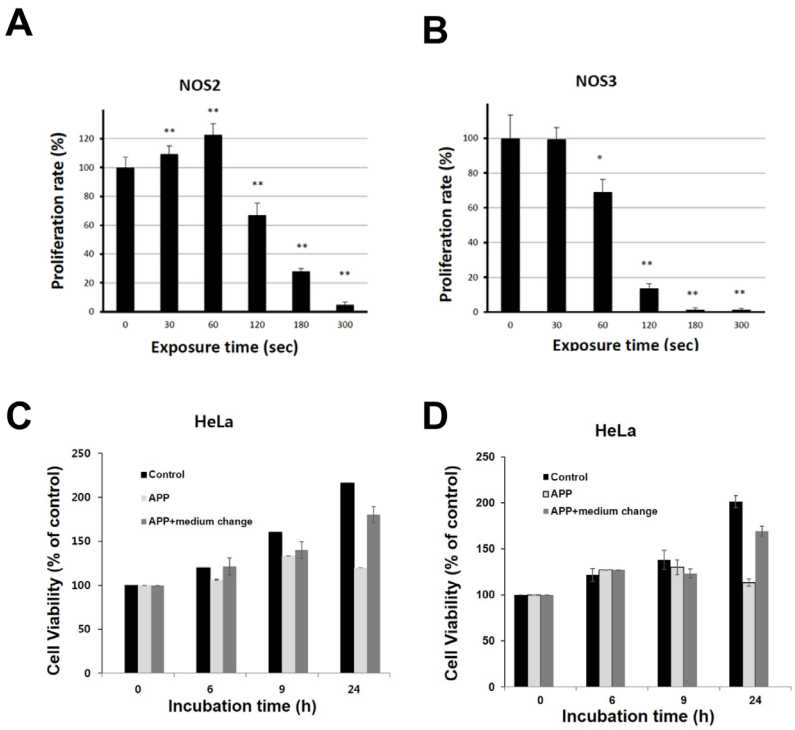

FIG. 2: *In vitro* antiproliferative effect of NTAPP-activated medium**. (a, b)** Medium irradiated with NTAPP for different exposure time (sec) as in the graph was incubated with ovarian cancer cell lines NOS2 and NOS3 for 24 h. Cell viability was assayed and the percentage of surviving cells relative to controls were plotted for **(a)** NOS2 and **(b)** NOS3. (Utsumi *et al.*,[16] open access). **(c, d)** HeLa cells exposed with 5 V input NTAPP for 30 s every h for 10 times and further incubated for 15 h were used as a positive control (APP). Unexposed to NTAPP, were used as a negative control (Control). Incubation time indicates the time after initial exposure to NTAPP or NTAPP-treated medium. Viable cells were quantified with MTT assays and the data are shown as mean ± SEM from three independent experiments. **(c)** Medium was exposed with 5 V input NTAPP for 30 s. The medium of HeLa cells were switched to NTAPP-treated one every h for 10 times (9 h) without direct NTAPP exposures and cells were further incubated for 15 h (24 h). **(d)** After HeLa cells were exposed every hour with NTAPP for 30 s, the medium was changed to a NTAPP-untreated fresh medium every hour and cells were further incubated for 15 h.

VI. HIGHLY PREFERENTIAL ANTIPROLIFERATIVE EFFECT OF NTAPP ON P53-DEFICIENT CANCER CELLS

The DNA damage checkpoint pathway is important to maintain the genomic stability of the cell. DNA damage caused by several different occasions are known to activate ATM and ATR kinases, which in turn activate kinase chk1 and chk2, further triggering DNA repair pathways and the cell cycle arrest (Fig. 4A). If DNA damage is too

TABLE 3. Evidence that ROS and RNS Generated by Nonthermal Atmospheric-Pressure Plasma are Responsible for Apoptosis

Cancer Cell Name	Cell Type	Mechanism	Reference
HeLa HCT116 p53+/+ HCT116-E6 p53-/-	Human adenocarcinoma Human colorectal carcinoma	ROS	Ma *et al*., 2014, *PLoS One*[10]
NOS2, NOS3	Human epithelial ovarian carcinoma cell	ROS	Utsumi *et al*., 2013, *PLoS One*[16]
HeLa	Human cervical carcinoma cell	ROS	Ahn *et al*., 2011, *PLoS One*[50]
HepG2	Human hepatocellular carcinoma cell	ROS and RNS	Yan *et al*., 2012, *Plasma Process and Polymers*[48]
A2058	Human melanoma cell line	ROS	Sensenig *et al*., 2010, *Annals of Biomedical Engineering*[51]
U87MG, HCT116	Glioblastoma cancer cell, Human colorectal cancer cell	ROS	Vandamme *et al*., 2012, *International Journal of Cancer*[14]
Mel-RM, Mel-007, Mel-JD	Human melanoma cell	ROS	Ishaq *et al*., 2014, *Molecular Biology of the Cell*[11]
FaDu, SNU1041, SCC, SNU899, HN9	Human hypopharynx squamous carcinoma cell, Human laryngeal squamous carcinoma cell, Human parotid gland carcinoma cell	ROS	Kang *et al*., 2014, *Cell Death and Disease*[23]

severe to be fixed, cells undergo apoptosis. The p53 tumor suppressor protein plays an essential role in the DNA damage checkpoint to maintain genomic stability in mammals. When cells are subjected to various genotoxic and cellular stresses such as oxidative stress, hypoxia, radiation, or chemotherapeutic drugs, p53 is activated, and its ubiquitin-dependent degradation is blocked, leading to an accumulation of active p53 transcription factor.[2] Activated p53 regulates cell cycle arrest, activation of antioxidants and DNA repair, and apoptosis by affecting the expression of its target genes, including the cyclin-dependent kinase (CDK) inhibitor *p21/WAF1* and genes involved in cell death, such as *BAX, PUMA, NOXA,* and *Fas*.[27,28] When cells are exposed to oxidative stress, p53 also activates the transcription of sestrin, glutathione peroxidase (GPX), and aldehyde dehydrogenase (ALDH), thus playing a pivotal role

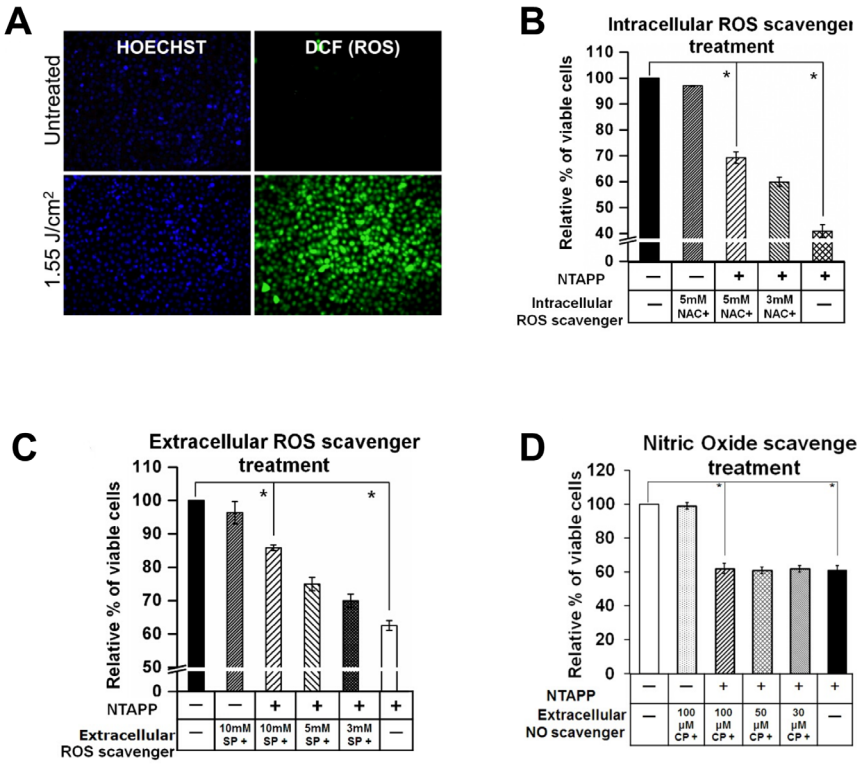

FIG. 3: Antiproliferative roles of ROS generated by NTAPP exposures. **(a)** H_2DCFA was used as a florescence dye to detect intracellular ROS by fluorescence microscopy. H_2DCFA was added to culture dishes of mammalian breast epithelial cells (MCF10A) and cells were treated with the indicated dose of NTAPP. One hour after plasma treatment, intracellular ROS was detected through a florescence microscopy. (Kalghatgi et al.,[22] open access). **(b, c, d)** HeLa cells pretreated with different concentrations of **(b)** NAC (untreated, 3 mM, 5 mM), **(c)** sodium pyruvate (SP, untreated, 3, 5, 10 mM), **(d)** a NO scavenger, carboxy-PTIO (0, 30, 50, 100 μM), were exposed with 5 V input NTAPP for 30 s every h 10 times and further incubated for 15 h. The relative percentages of viable cells are shown comparing the initial cell number prior to exposure and incubation as 100%. Viable cells were quantified with MTT assays, and data are shown as mean ± SEM from three independent experiments. $p < 0.05$ (*) indicates a significant difference compared with the control (Ma *et al.,*[10] open access).

in maintaining redox balance and genomic stability under oxidative stress.[29,30] Mutation of the p53 gene or disruption of the pathway that leads to p53 activation have been frequently observed in most types of human cancer.[31] More than 50% human cancer cells have been reported to have p53 mutations by which the function of p53 is defective or deleted.

The p53-dependent induction of apoptosis in response to genotoxic damage is an important aspect of tumor suppression. Thus, the loss of p53 in human cancers contrib-

utes to aggressive tumor behavior and often promotes resistance of cancer cells to radiation and chemotherapeutic drugs. For example, treatment of $p53^{+/+}$ mouse thymocytes with radiation results in apoptosis, whereas $p53^{-/-}$ thymocytes are resistant. Similarly, $p53^{+/+}$ mouse embryonic fibroblasts transformed by adenoviral E1A protein and Ha-ras oncogene undergo apoptosis in response to γ-irradiation or chemotherapeutic agents, but $p53^{-/-}$ fibroblasts are resistant to both treatments.[32] In addition, some p53 mutations in cancers suppress the function of p73, which induces apoptosis through a p53-independent mechanism.[33] Thus, the common loss of p53 function in cancer cells presents a major limitation for anticancer therapies. Actually, the lack of functional p53 usually allows cancer cells to resist γ-irradiation or chemotherapeutic agents.[32]

When our group monitored the antiproliferative effect of NTAPP in various cancer cells, we observed that p53-deficient cancer cells (DLD-1, H1299, HT29, and HCT15) were much more sensitive to NTAPP than p53-proficient cancer cells (LoVo, MES-SA, HepG2, and RKO) (Fig. 4B). To further verify the reverse correlation between the function of p53 and the antiproliferative effect of NTAPP in cancer cells, we compared the number of viable cells after NTAPP exposure in p53-negative HT29 cells transfected with p53-expressing vector or with a vector-only control. The relative number of viable cells in HT29 expressing the wild-type p53 was increased to the level observed in other p53-proficient cancer cells (Fig. 4C). In short, these observations confirm that the antiproliferative effect of NTAPP is highly preferential toward p53-deficient cancer cells. In addition, our results demonstrate NTAPP exposures lead to G1 cell-cycle delay in a p53-dependent manner prior to apoptosis in p53-proficient cells.[10] Yan et al. also demonstrated that the percentage of cells arrested at G2/M phase increased after plasma treatment,[34] supporting the idea that cell cycle arrest or delay by plasma treatment is a general cellular response. Altogether, these observations let us propose that NTAPP exposures induces DNA DSB and activate p53 to delay the cell cycle at G1, leading to apoptosis in p53-proficient cells, while NTAPP expedites p53-independent apoptosis in p53-deficient cells.

The JNK/p38 mitogen-activated protein kinase (MAPK) pathway has also been proposed to activate apoptosis by NTAPP treatment (Fig. 4D). Kang et al. demonstrated that NTAPP induced apoptosis of head and neck cancer cells through MAPK-dependent mitochondrial ROS.[23] They showed that JNK and p38 become activated only in the cells treated with NTAPP, which lead to the cleavage of procaspase-3 and PARP for apoptosis. However, an antioxidant NAC, JNK inhibitor SP600125, and p38 inhibitor SB203580 blocked the phosphorylation of JNK and p38, leading to the decreased cleavage of caspase-3 and PARP.[23] Similarly, Ishaq et al. proposed that ROS can induce apoptosis through the activation of JNK/p38.[11]

VII. ANTIPROLIFERATIVE EFFECT OF NTAPP ON CANCER CELLS RESISTANT TO CURRENT CANCER THERAPIES

The main hurdle in chemotherapy is overcoming the drug-resistance of cancer cells. Because NTAPP has a selective antiproliferative effect on cancer cells and may be a po-

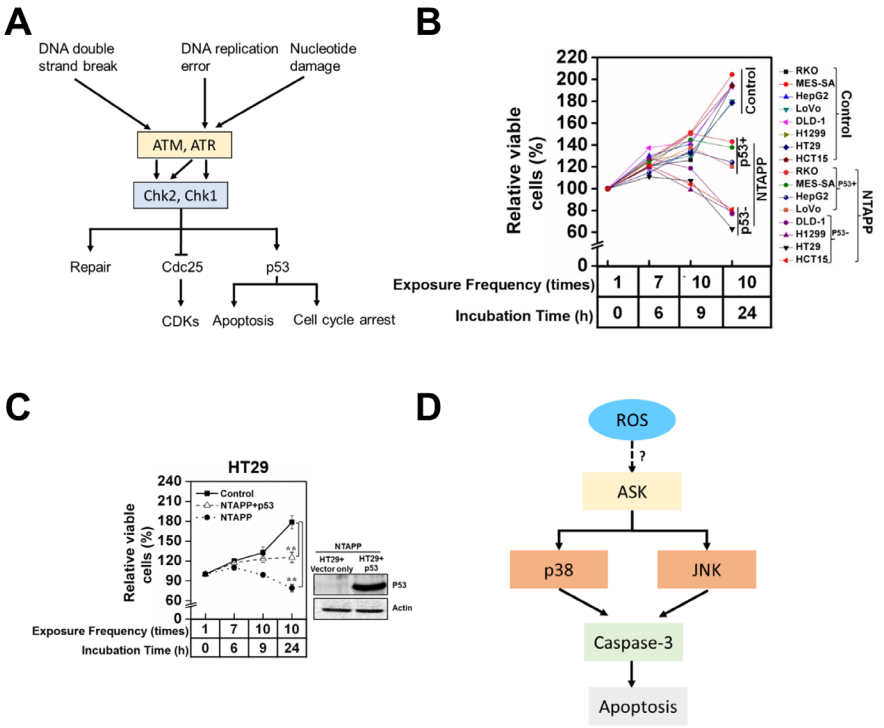

FIG. 4: The molecular mechanism of NTAPP-induced cell apoptosis.**(a)** The typical DNA damage signaling pathway in mammalian cells. The ATM and ATR kinases become activated by various DNA damages, which in turn activate Chk1 and Chk2 kinases. The activated Chk1 and Chk2 further activate DNA damage repair pathways and arrest cell cycle through Cdc25 and p53 (modified from Lodish *et al.*[46]). **(b)** Various cancer cells were exposed with 5 V input NTAPP for 30 s every hour 10 times and further incubated for 15 h. The relative percentages of viable cells were plotted together after the same NTAPP exposures in p53-proficient cells (RKO, MES-SA, HepG2, G361, LoVo) and p53-deficient cells (DLD-1, H1299, HT29, HCT115). **(c)** p53-deficient HT29 cells were transfected with pcDNA-p53-HA, and the expression of p53 in HT29 was verified by a western blot assay (shown on the right side) with actin as a loading control. The relative percentages of viable cells in HT29 and p53-transfected HT29 cells were plotted after the same NTAPP exposures. **(d)** JNK/p38 pathway was proposed as a mechanism of NTAPP-induce apoptosis.

tential anticancer therapy, the antiproliferative effect of NTAPP needed to be examined in drug-resistant cancer cells. We demonstrated that the NTAPP exposure also induces apoptosis in two different doxorubicin-resistant cancer cell lines: human colon carcinoma-derived HCT15/CL02 and human uterus sarcoma-originated MES-SA/dx5 cells.[10] By indirectly applying NTAPP-exposed medium to chronic paclitaxel and cisplatin-resistant ovarian cancer cells, Utsumi et al. also showed that NTAPP may induce apop-

totic death in chemo-resistant cancer cells.[16] Moreover, NTAPP might also be used in combination with other cancer therapies to increase anticancer efficiency. For example, Brelle et al. showed that the apoptotic cell death by NTAPP improved in combination with gemcitabine, a nucleoside analog used as chemotherapy.[13] These observations support the merit of NTAPP as a potential anticancer therapy. These qualities of NTAPP are important because chemotherapeutic drug-resistance of cancer cells represents a great obstacle to the efficacy of anticancer therapies.

VIII. CONCLUSION

NTAPP has been suggested as a new cancer therapy. To develop NTAPP as a potential cancer therapy, its selectivity to cancer cells must first be documented. In this review, we have focused on the selective effect of NTAPP on the apoptosis of cancer cells, and we have presented the known molecular mechanism of NTAPP-induced apoptosis.

More than 50% of human tumors have a mutation that affects the function of the tumor suppressor p53.[35] Inheriting a mutated p53 allele increases cancer susceptibility and reduces sensitivity to anticancer therapies. In general, cells with defective p53 are relatively resistant to apoptosis and, thus, to chemotherapeutic agents. In this regard, the finding that NTAPP exerts a highly preferential antiproliferative effect on p53-deficient cancer cells suggests a great potential of NTAPP as a new anticancer therapy.

Why are cancer cells, and especially p53-deficient cancer cells, more sensitive to NTAPP? We and many others have shown that the antiproliferative apoptotic effect of NTAPP is due to increased intracellular ROS. ROS induces DNA damage and activates p53 to delay cell cycle and apoptosis in p53-proficient cells.[36–38] Ma et al. showed that the hypersensitivity of p53-deficient cancer cells to NTAPP is correlated with the lack of p53-dependent cell cycle arrest or delay.[10] Compelling evidence indicates that cancer cells, especially p53-defective cells, have higher levels of endogenous ROS than normal cells due to oncogene hyperactivation and aberrant metabolism.[37,39,40] Thus, cancer cells have to survive in the higher intrinsic oxidative stress environments than normal cells, and they become more vulnerable to damage by ROS-generating agents.[41–43] In fact, some research groups have attempted to modulate redox homeostasis to selectively induce apoptosis in cancer cells without significant damage to normal cells.[39,44,45] Thus, the high selectivity of NTAPP toward cancer cells might be attributable to the differential sensitivity of cancer cells versus primary cells to increased intracellular ROS by NTAPP. The preferential antiproliferative effect of NTAPP toward p53-deficient cells might also be due to the defective redox homeostasis in p53-defective cells[10] because wild-type p53 regulates the upregulation of several antioxidant genes.[29,30] Alternatively, Volotskova et al. hypothesized that the increased sensitivity of cancer cells to plasma treatment is due to the difference in the distribution of cancer cells and normal cells within the cell cycle.[8]

Which particular component of plasma induces the increase of intracellular ROS is not yet clear. NTAPP could directly and/or indirectly cause changes in ROS components and composition. The physiological effect of NTAPP on cells could be due to a com-

bination of the interactions between various electromagnetic radiations with ions, electrons, and reactive chemical species in the cell. To develop NTAPP as an effective anticancer therapy, further studies into the chemistry and kinetics of plasma are necessary to understand the physiological mechanism of NTAPP and to increase its reproducibility.

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