

Comparative Effects of Non-Thermal Atmospheric Pressure Plasma on Migration and Invasion in Oral Squamous Cell Cancer, by Gas Type

Sung Un Kang^{1*}, Seong Jin Seo^{1*}, Yeon Soo Kim², Yoo Seob Shin¹, Yoon Woo Koh³,
Chang Min Lee⁴, Sang Sik Yang⁴, Jong-Soo Lee⁵, Eunpyo Moon⁵, Hami Kang⁶,
Jeong Beom Ryeo⁷, Yuijina Lee⁷, and Chul-Ho Kim¹

¹Department of Otolaryngology, Ajou University School of Medicine, Suwon;

²Department of Otorhinolaryngology, Konyang University College of Medicine, Daejeon;

³Department of Otorhinolaryngology, Yonsei University College of Medicine, Seoul;

⁴Department of Electrical and Computer Engineering, Ajou University, Suwon;

⁵Department of Life Science, Ajou University, Suwon;

⁶Ajou University, Suwon;

⁷ICD Co., Ltd., Anseong, Korea.

Purpose: The fourth state of matter, plasma is known as an ionized gas with electrons, radicals and ions. The use of non-thermal plasma (NTP) in cancer research became possible because of the progresses in plasma medicine. Previous studies on the potential NTP-mediated cancer therapy have mainly concentrated on cancer cell apoptosis. In the present study, we compared the inhibitory effect of NTP on cell migration and invasion in the oral squamous cancer cell lines.

Materials and Methods: We used oral squamous cancer cell lines (SCC1483, MSKQLL1) and different gases (N₂, He, and Ar). To investigate the mechanism of plasma treatment, using different gases (N₂, He, and Ar) which induces anti-migration and anti-invasion properties, we performed wound healing assay, invasion assay and gelatin zymography.

Results: The results showed that NTP inhibits cancer cell migration and invasion of oral squamous cancer cell. In addition, focal adhesion kinase expression and matrix metalloproteinase-2/9 activity were also inhibited.

Conclusion: The suppression of cancer cell invasion by NTP varied depending on the type of gas. Comparison of the three gases revealed that N₂ NTP inhibited cell migration and invasion most potently via decreased expression of focal adhesion kinase and matrix metalloproteinase activity.

Key Words: Non thermal atmospheric pressure plasma, oral squamous cancer cell, focal adhesion kinase, matrix metalloproteinase-2/9, paxillin

INTRODUCTION

Oral squamous cell carcinoma (OSCC) accounts for ~3% of all cancer diagnoses, and is one of the most common head-and-

neck cancer (HNC).¹ There has been a lot of progress in OSCC treatment protocols including surgery, radiotherapy and chemotherapy, nevertheless, the long-term survival rate of patients with OSCC unchanged over the past decade.^{1,2} Therefore, many

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Corresponding author: Dr. Chul-Ho Kim, Department of Otolaryngology, Ajou University School of Medicine, 164 World cup-ro, Yeongtong-gu, Suwon 16499, Korea.
Tel: 82-31-219-5269, Fax: 82-31-219-5264, E-mail: ostium@ajou.ac.kr

*Sung Un Kang and Seong Jin Seo contributed equally to this work.

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studies have been conducted for OSCC treatment. In these studies, we found that a combination of inter-reliant processes, including cell migration, invasion, surface adhesion, and extracellular matrix (ECM) degradation by matrix metalloproteinases (MMPs), resulted in an impact on the invasive and metastatic mechanism by complicated mechanisms.^{3,4} Tumor metastasis is one of major cause of mortality in cancer patients.⁵ Thus, preventing cancer cell migration and invasion, which induce tumor metastasis, is crucial for cancer treatment.

Non-thermal plasma (NTP) is an ionized gas consisting of electrons, ions, neutral atoms, radicals, and UV photons; also called the fourth state of matter after solid, liquids and gases.⁶ NTP is known as a novel treatment method for various cancers including lung cancer, melanoma and colorectal cancer,⁷⁻¹⁰ and we previously reported that NTP induced apoptosis in HNC via the ATM/p53 signaling pathway,¹¹ and caused cell death through mitochondrial reactive oxygen species by mitogen-activated protein kinase.¹² It has also been reported that NTP suppresses the invasion of thyroid papillary cancer cell through cytoskeletal modulation.⁴ In addition, we reported that NTP induced delayed tumor invasion and growth arrest in colorectal and thyroid cancer cells.^{4,12} The anti-cancer effect of NTP in these instances was explained mainly by apoptosis due to the increase in reactive oxygen species.¹³

Many studies showed that biological effects of NTP vary by their gas types, their combinations, treatment conditions, and treated cell types.¹³⁻¹⁷ Different plasma gas sources were used depending on various research groups and indications including anti-infection,^{18,19} wound healing,²⁰ and anticancer treatment.^{3,12} However, the inhibitory effects of NTP composed of different gases on cancer migration and invasion have not been directly compared until now. The purpose of this study is to compare the emission spectra between N₂, He, and Ar, and the NTP suppressing effects on cell migration, cell invasion and apoptotic cell death in OSCC according to the type of gas. As far as we are aware of this is the first study comparing the anti-cancer effects on cell migration and invasion according to NTP gas type.

MATERIALS AND METHODS

Cell lines and reagents

Two HNC cell lines originating from human oral cancer (MSKQLL1, SCC1483) were provided by Prof. Se-Heon Kim (Yonsei University, Korea). MSKQLL1 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM): Nutrient Mixture F-12 (DMEM/F12; GIBCO, Carlsbad, CA, USA). whereas SCC1483 cells were cultured in Minimum Essential Medium (MEM; GIBCO, Carlsbad, CA, USA). The cells were grown in MEM with 10% fetal bovine serum and 1% penicillin-streptomycin, respectively. The cultured cells were maintained at 37°C in a humidified atmosphere with 5% CO₂/95% air.

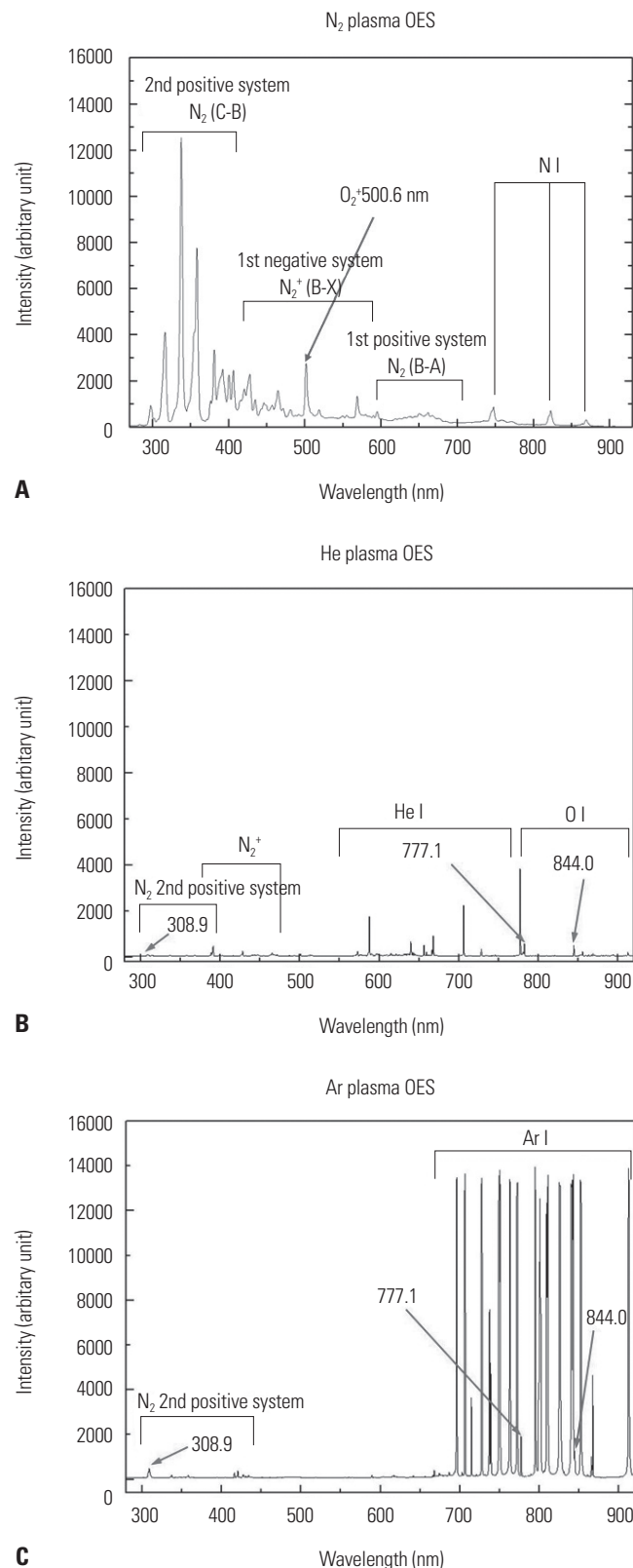


Fig. 1. Optical emission spectrum (OES) of plasma jet according to gases (A) OES of NTP induced by N₂ gas, (B) He gas, and (C) Ar gas. NTP, non-thermal plasma.

Experimental system specifications and NTP treatment

We used a micro-nozzle plasma jet system. The microplasma jet nozzle is made up of a plasma generation module and its cases. The schematic view of its nozzle is shown on Supplementary Fig. 1A (only online). The main components of plasma generation module are a Ni-Co alloy electrode, a glass insulator, and an electrode ring. The Ni-Co alloy electrode and glass insulator were fabricated simultaneously by micromachining technology. Supplementary Fig. 1B (only online) shows the fabrication process of Ni-Co alloy electrode. A Cr/Au film was deposited on a glass wafer as a seed layer for electroplating. The 105 holes were arrayed along concentric circles on the Ni-Co alloy electrode and glass insulator, and the diameter and the depth of holes were 300 μm and 100 μm , respectively. Cells were grown up to 90% confluence and exposed to NTP (He, Ar, and N_2) 3 cm away from the nozzle for 1, 3, and 5 min. The gas flow rate

was maintained at 8 L/min, and voltages were 15 KV and 20 KHz, respectively. We developed and produced NTP with a designed dielectric barrier discharge type to generate a homogeneous NTP jet for biomedical research applications, as described previously.^{21,22}

Annexin V and propidium iodide (PI) analysis of apoptosis cells

Quantitative apoptotic cell death by plasma was detected using the Annexin V-propidium iodide (PI) apoptosis detection kit I according to the manufacturer's protocol (BD Biosciences, Bedford, MA, USA), as described previously.¹² The cells were treated with various gas device (Ar, N_2 , and He) NTP for 1, 3, and 5 min and then incubated further for 24 h. The cells were harvested, washed with cold phosphate-buffered saline (PBS), and stained with Annexin V-fluorescein isothiocyanate and PI at room temperature for 15 mins in the dark. The early and late

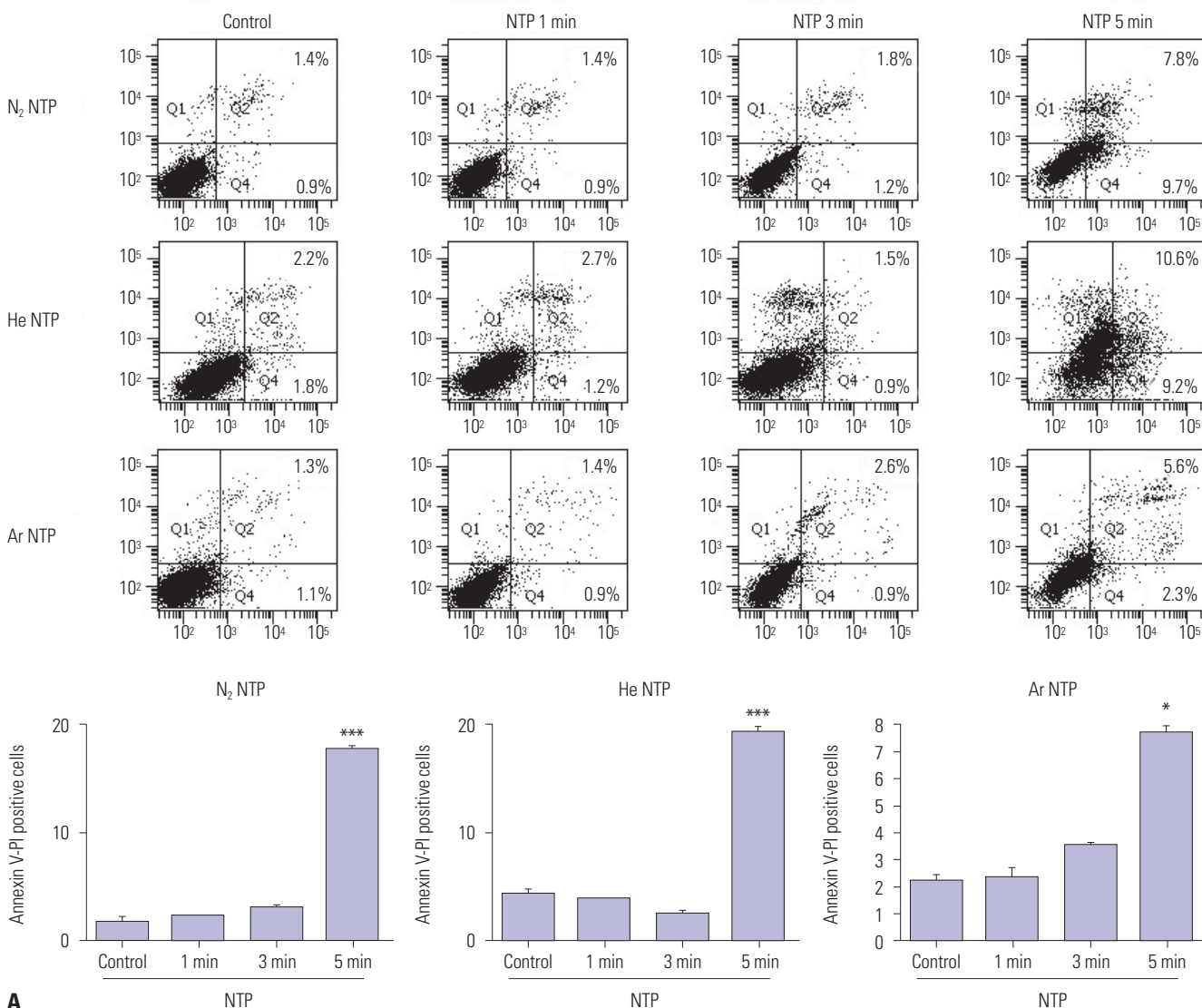


Fig. 2. Cell death after 1, 3, and 5 min NTP treatment according to gases (N_2 , He, or Ar). (A) Annexin V-PI assay and quantification of SCC1483. Bar graph: mean \pm SD of 3 independent experiments. * $p < 0.05$, *** $p < 0.001$. NTP, non-thermal plasma; PI, propidium iodide.

apoptosis were quantified according to the manufacturer's instructions. Apoptosis was detected using a FACS Aria system (BD Biosciences), with the excitation and emission settings of 488 and 530 nm, respectively.

Scratch wound healing assay

For wound healing assay, inducible MSKQLL1 and SCC1483 cells were plated on 12-well plates and grown to confluency (>90%), followed by serum starvation for 24 h. Wounds were generated by using a sterile 200- μ L pipette tip and washing with PBS. The cells were then exposed to various gases (N_2 , Ar, and He) of NTP for 3 min. Each experiment was performed in triplicate. The wound on the captured image was automatically recognized and measured by Metamorph[®] NX image software (Molecular Devices, Sunnyvale, CA, USA) and eluate of crystal violet staining was measured under a light microscopy.

Transwell invasion assay

The invasion ability of each cancer cell line was evaluated using Transwell (24-well) chambers (pore size 8 μ m, Costar, Cambridge, MA, USA), as described previously.⁷ Initially, type I collagen (8 μ g/filter) was dissolved in 100 μ L of medium and poured into the upper part of the polyethylene filter (pore size, 8 μ m). The wells were coated overnight in a laminar flow hood. Then, 1×10^4 cells (in 100 μ L of culture medium) were added to the top of the filter in the upper transwell chamber. The chamber was incubated for 24 h in 5% CO_2 at 37°C. Finally, attached cells in the lower section (invading cells) were stained with hematoxylin and eosin (H&E) and counted in four representative fields by light microscopy ($\times 200$ magnification).

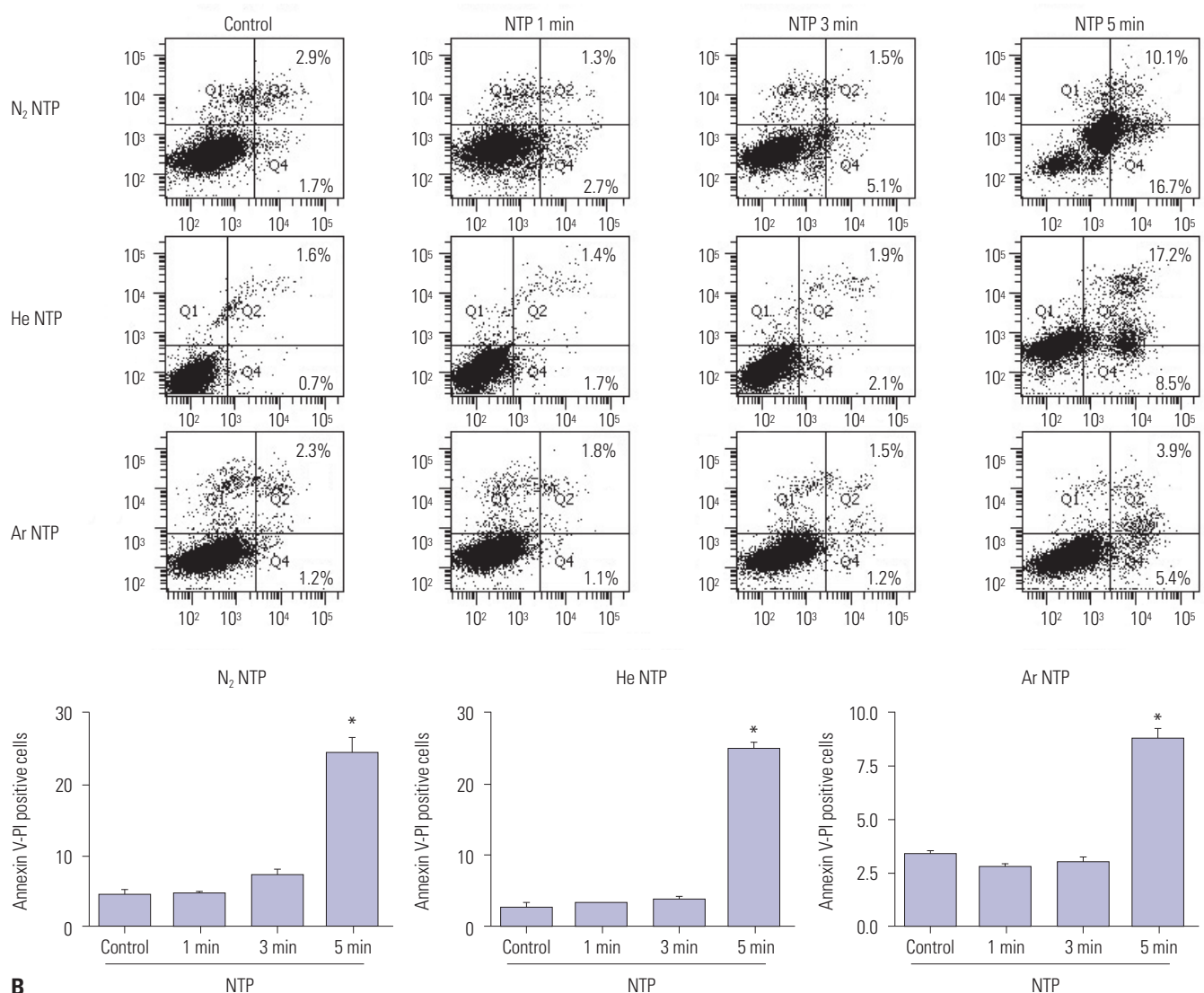
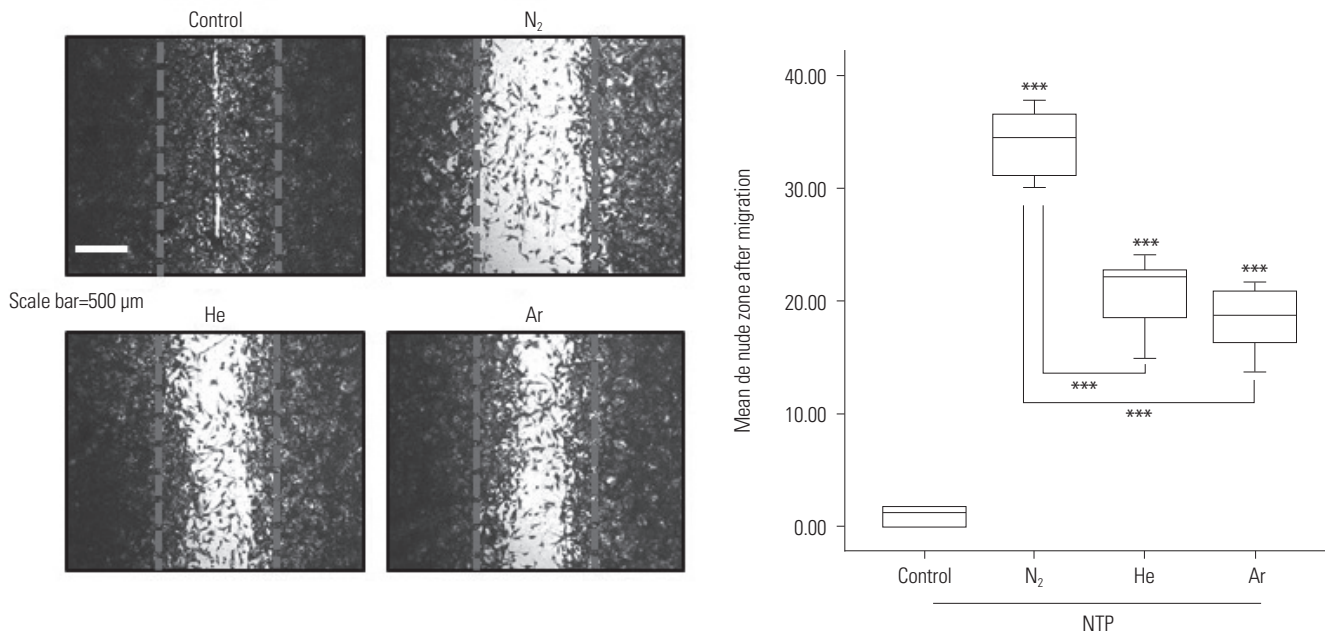


Fig. 2. Cell death after 1, 3, and 5 min NTP treatment according to gases (N_2 , He, or Ar). (B) Annexin V-PI assay and quantification of MSKQLL1. Bar graph: mean \pm SD of 3 independent experiments. * $p < 0.05$. NTP, non-thermal plasma; PI, propidium iodide.

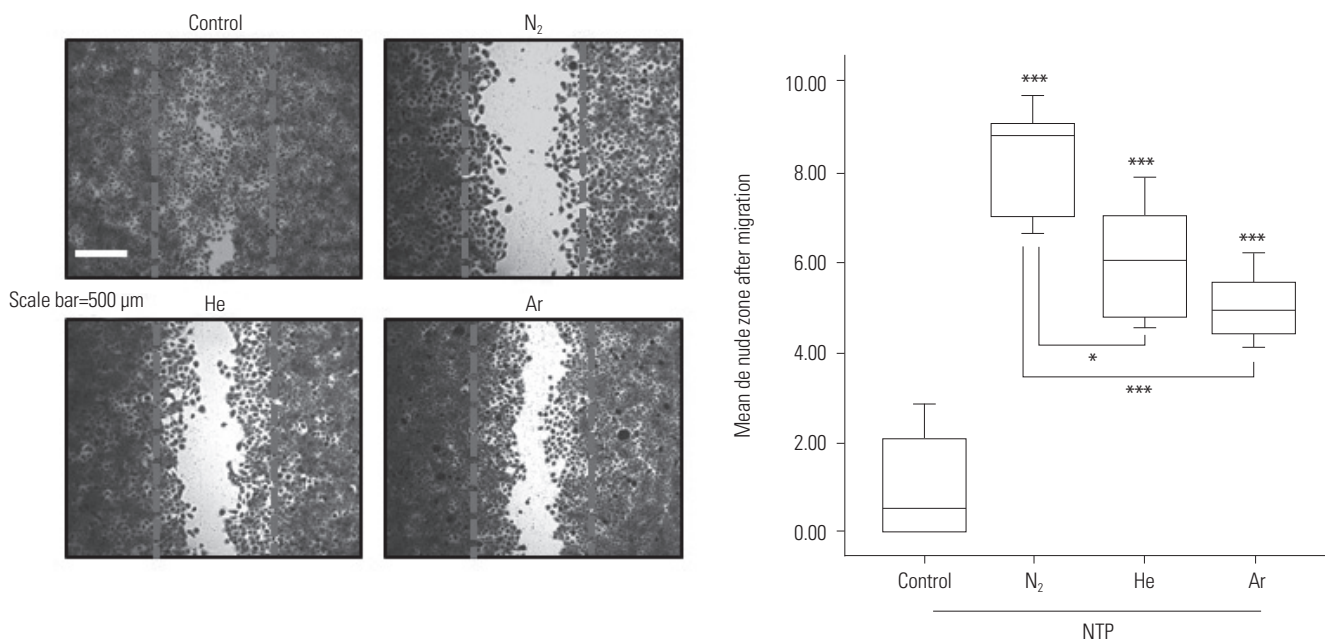
Gelatin zymogram assay

MMP-2/9 activity was examined using gelatin zymography as described previously.¹⁷ The cells were treated with He, Ar, or N₂ plasma for 5 min and incubated for an additional 24 h. The supernatant (100 μL) from each sample was mixed with 1 μL of 100 mM APMA (4-aminophenylmercuric acetate, Sigma-Aldrich, St. Louis, MO, USA) and the samples were activated for 1 h at 37°C incubator. Next, each sample was placed in sample buffer (without mercaptoethanol) for 10 min and electropho-

resed in sodium dodecyl sulfate (SDS) page gelatin gels at 125 V for 120 min at room temperature using a Novex Xcell II system (Thermo Scientific, Waltham, MA, USA). The gels were incubated in renaturation buffer for 60 min at room temperature, followed by incubation for 18 h in 100 mL of Novex zymogram developing buffer (Thermo Scientific) at 37°C with orbital shaking. The gels were then stained for 3 h with Coomassie brilliant blue. After decolorization in 400 mL of methanol, 100 mL of acetic acid, and 500 mL of distilled water; images were taken us-



A SCC1483/plasma 3 min



B MSKQLL1/plasma 3 min

Fig. 3. Wound healing assay of 3 min NTP treatment according to gases (N₂, He, or Ar) on (A) SCC1483 and (B) MSKQLL1. Data represents mean ± SD of three independent experiments. **p* < 0.05, ****p* < 0.001. NTP, non-thermal plasma.

ing an image analyzer.

Western blot

For Western blot, reduced protein samples were lysed by RIPA buffer containing 150 mM NaCl, 1.0% nonidet-P 40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0, and protease inhibitor cocktail (Roche Applied Science, Vienna, Austria; pH 7.4), as described previously.¹² The following antibodies were used for Western blot analysis: anti-focal adhesion kinase (FAK), -integrin, -Paxillin, and - α -tubulin (1:1000; Cell Signaling

Technology, Danvers, MA, USA).

Immunocytochemistry

After culture on a microscope cover glass (Thermo Scientific), the cells were treated with He, Ar, and N₂ of NTP, and incubated for an additional 24 h. The cells were fixed with 4% formaldehyde and blocked in bovine serum albumin in 5% PBS for 45 min. Slides were then incubated with a polyclonal rabbit anti-p-FAK antibody (1:50; Cell Signaling Technology) for 2 h, washed with PBS and incubated with an Alexa 546-labeled goat anti-

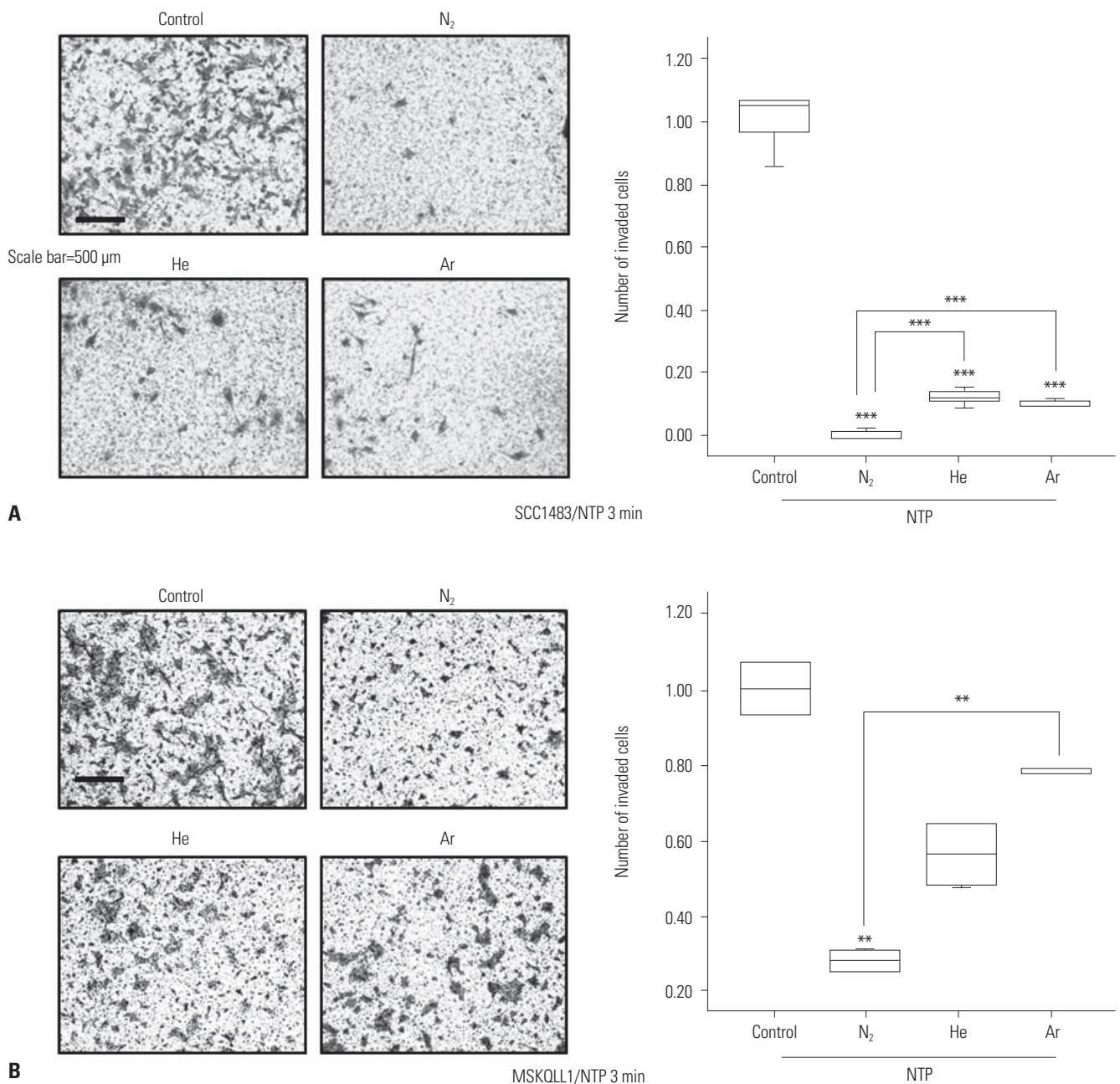


Fig. 4. Invasion assay of 3 min NTP treatment according to gases (N₂, He, or Ar) on SCC1483 (A) and MSKQLL1 (B). Data represent mean \pm SD of three independent experiments. ** p <0.01, *** p <0.001. NTP, non-thermal plasma.

rabbit antibody (1:250; Molecular Probe, Eugene, Oregon, CA, USA) for 45 min. After rinsing in PBS, Hoechst 33258 (Molecular Probe) was added to slides for 15 min to counterstain nuclei. Slides were washed with PBS, mounted with Vectashield (Vector laboratories, Inc., Burlingame, CA, USA), and then visualized using a fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

Statistical analyses

All data from three independent experiments are expressed as mean±SD. A $p < 0.05$ was considered statistically significant (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

The means for the different groups were compared using One-way analysis of variance, followed by post hoc Tukey's test, performed using SPSS 20.0 statistical software (SPSS Inc., Chicago, IL, USA).

RESULTS

The emission spectra of NTP are different depending on the type of gas

To estimate various species produced from the plasma jet, we performed optical emission spectrum analyses. Optical emission spectroscopy was conducted from 280 nm to 920 nm wavelengths by optical emission spectroscopy (SV 2100, K-MAC,

Daejeon, Korea) (Fig. 1), and the optical emissions of three gases were compared: N₂, He, and Ar from a NTP jet at 5 L/min. The N₂ emission spectrum (Fig. 1A) was settled mostly by the presence of nitrogen (N) species, including N₂ second (290–410 nm), first positive systems (600–700 nm) and N₂⁺ first negative system (410–600 nm). Moreover, considerably reactive radicals were tied to oxygen, such as oxygen ions (O₂⁺) at 500–600 nm and weak atomic N at 747, 822, and 868 nm, were observed. The Ar and He NTP emission spectra (Fig. 1B and C) showed that the respective plasma species tied to argon and helium were superior. From Ar and He NTP in the emission spectra, we detected hydroxyl radicals and excited oxygen atoms. However, the intensities of oxygen atoms in Ar and He NTP were weaker than in N₂ NTP. These findings imply that N₂ NTP would be more effective for biomedical applications than He or Ar NTP.

NTP by all types of gas induce apoptotic cell death in OSCCs

It has been previously demonstrated that NTP induce apoptotic cancer cell death.¹² Therefore, to estimate the effects of NTP on apoptotic cell death by different gas types, we measured annexin V-PI staining according to the processing time of each gas (Fig. 2). No significant SCC1483 and MSKQLL1 cell death was induced by 1 and 3 min NTP treatment with various gas sources (N₂, Ar, and He). However, significant cell death in both SCC1483 (Fig. 2A) and MSKQLL1 (Fig. 2B) cell lines was noted in all three

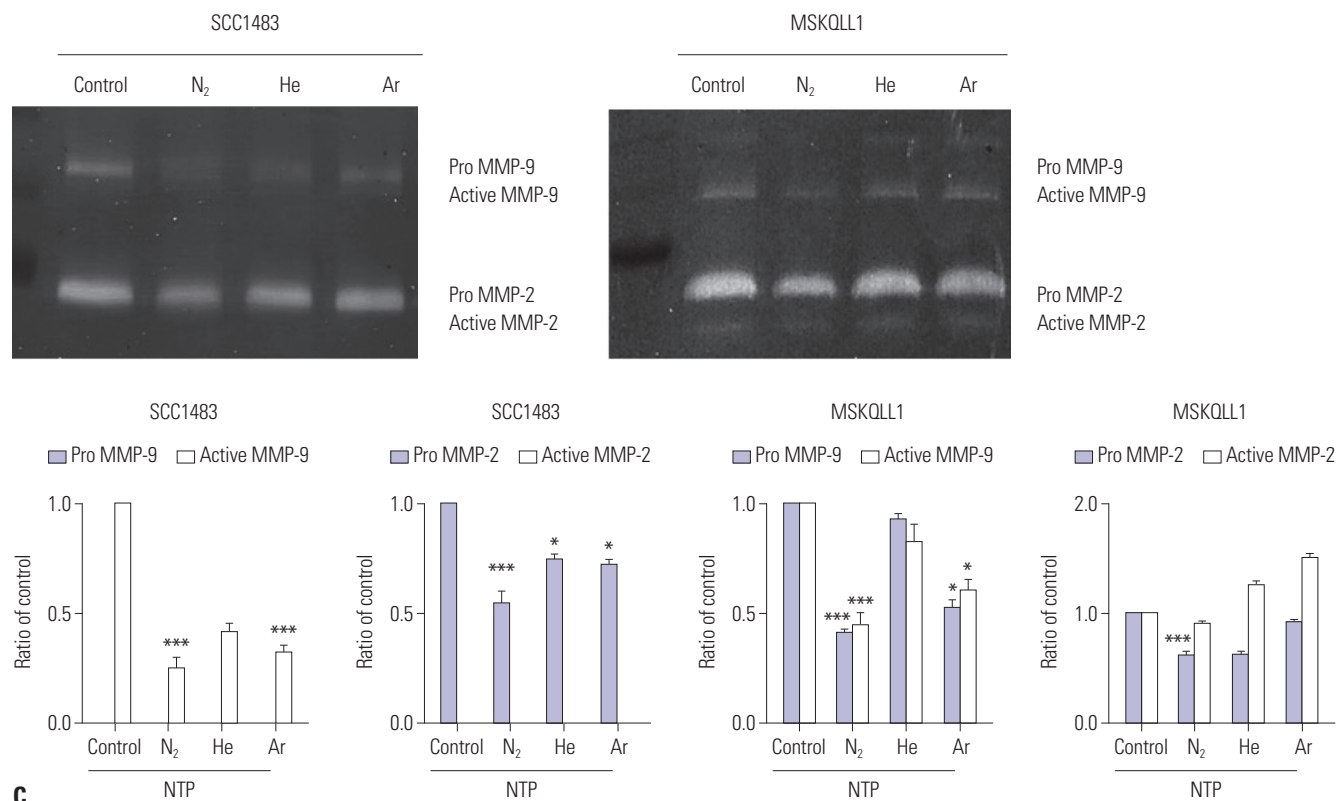


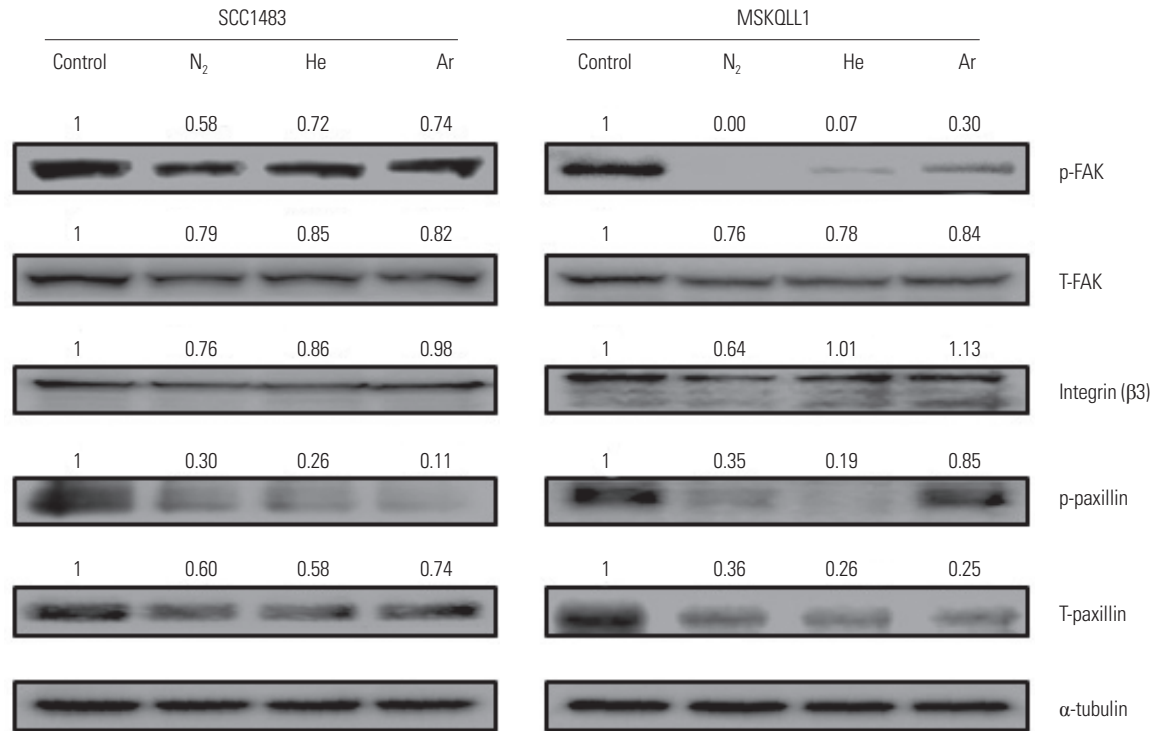
Fig. 4. (C) Gelatin zymography of 3 min NTP treatment according to gases (N₂, He, or Ar) for MMP-2/9 on SCC1483 and MSKQLL1. * $p < 0.05$, *** $p < 0.001$. NTP, non-thermal plasma; MMPs, matrix metalloproteinases.

gas plasmas after 5 min treatments.

N₂ NTP suppressed cell migration in OSCC more than other gas types

Our previous studies showed that NTP treatment with the mixture of O₂ and He induces cellular morphological change and reduces tumor cell migration.³ To estimate the effect of NTP

on cell migration in OSCC by gas type, we performed a wound-healing assay (Fig. 3): NTP treatment for 3 min was used, because NTP treatment for 5 min induced significant cancer cell death (Fig. 2). Thus, cells were exposed to N₂, He, and Ar NTP for 3 min and incubated for 24 h. Results showed that that NTP with all three gases significantly suppressed the migration of SCC1483 (Fig. 3A) and MSKQLL1 (Fig. 3B) cells across the de-



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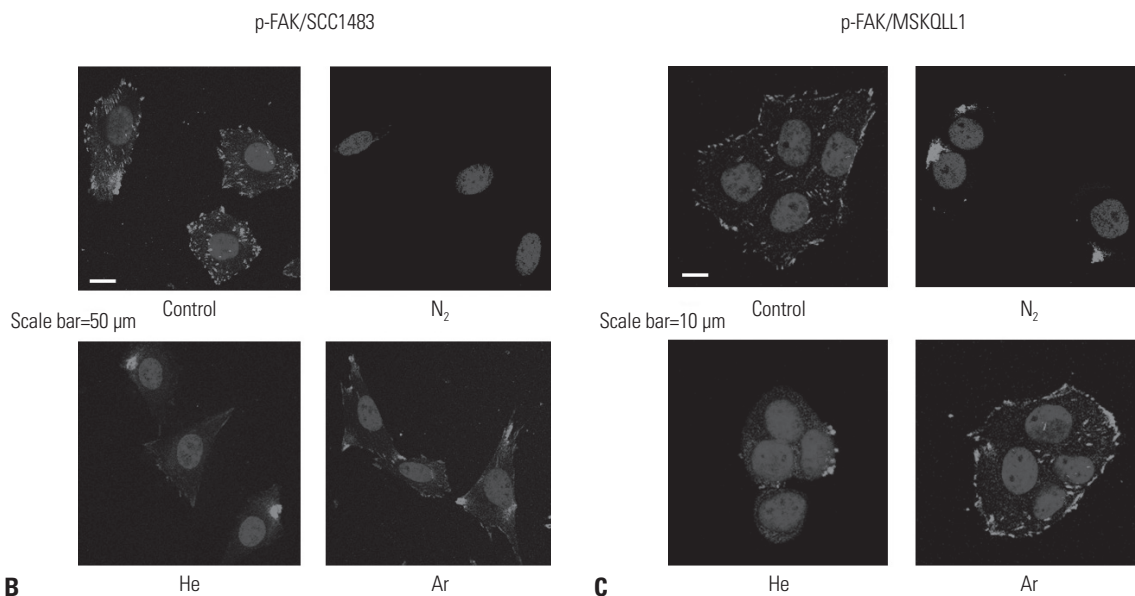


Fig. 5. (A) Western blots of p-focal adhesion kinase (FAK), FAK, Integrin (β3), p-paxillin, paxillin, and uncleaved caspase-3 after 3 min NTP treatment according to gases (N₂, He, or Ar). (B) Immunocytochemical assay for p-FAK after 3 min NTP treatment according to gases (N₂, He, or Ar) in SCC1483 and (C) MSKQLL1. Scale bar=10 μm. Each figure is representative of three experiments with triplicates. NTP, non-thermal plasma.

nuded zone compared with control group. Furthermore, N₂ NTP significantly suppressed the migration of both cell lines compared with He and Ar NTP.

N₂ NTP inhibits cell invasion in OSCC more than other gas types

To elucidate the suppressive effect of NTP induced by each gas on cellular invasion, we performed an invasion assay (Fig. 4). Twenty-four hours after incubation, H&E-stained OSCC cells were placed on the undersurface of the membrane. NTP treatment reduced the number of invading cells compared with gas-only treatment. It should be noted that N₂ NTP among N₂, He, and Ar NTP showed the most deterrent effect on cell invasion (Fig. 4A and B). In order to understand the mechanism of suppression effect of NTP on tumor invasion *in vitro*, we performed the gelatin zymography. Fig. 4C shows visible changes in MMP-2/9 activity in NTP-treated SCC1483 and MSKQLL1 cells (Fig. 4C), with the most significant reduction by N₂ NTP among the gases.

N₂ NTP downregulates protein expressions of FAK, integrin and paxillin in OSCC

FAK, integrin, and paxillin are well known proteins involved in cell migration and invasion.²³ Thus, to elucidate the suppressive effect of NTP on migration, the expressions of T-FAK, p-FAK, integrin β 3, t-paxillin, and p-paxillin were examined by western blot analysis. As shown in Fig. 5A, the levels of p-FAK and integrin β 3 in N₂ NTP treated cells were significantly lower than those in He and Ar NTP in both cell lines. Moreover, immunocytochemistry confirmed that intracellular distribution of p-FAK underwent the most significant alteration in N₂ NTP-treated SCC1483 and MSKQLL1 cells (Fig. 5B and C), respectively.

DISCUSSION

Recently, many research groups are actively involved in plasma medicine, and NTP has already been used widely in industrial and medical applications. According to previous studies, NTP is a novel therapeutic method which can induce cell death and inhibit cell migration and invasion in various cancers including HNC,^{2,11,12} and many studies showed the anticancer effect of NTP via various mechanisms including cell cycle arrest and apoptosis induction.^{7,8,10} We previously, reported that NTP exert a tumor suppression by inhibiting migration and invasion of cancer cell.⁴ However, the results were not reproducible due to different experimental system conditions.^{11,12,24} In this study, we compared the NTP effects on cancer cell survival, migration, and invasion with different gases (N₂, He, and Ar). To our best knowledge, it is the first time to explore optimized NTP condition which induces the best anticancer effect in OSCC cells.

To optimize the time of NTP treatment, we performed an-

nexin V-PI staining after 1, 3, and 5 min of NTP treatment using N₂, Ar, and He as gas sources. Five min NTP treatment by all the three gases induced significant apoptotic cell death in both SCC1483 and MSKQLL1 cell lines. Therefore, to clearly evaluate the effect on cancer cell migration and invasion, we selected 3 min NTP treatment condition.

MMPs are main members of proteases to degrade the ECM components. MMP activity is associated with tumor growth and metastasis. MMP-2/9 are highly expressed in colon, lung, prostate and breast cancer, and increased MMP-2/9 activity is due to the activation of FAK/ERK signaling.²⁵⁻²⁷ Control of MMP is closely related to reducing the cancer cell invasion. In the present study, to compare the NTP suppression effect on tumor invasion by gas type *in vitro*,²³ gelatin zymography was performed to confirm the MMP-2/9 activity, and the results of the zymography showed that NTP treatment by all the gases reduced MMP-2/9 expressions. In particular, N₂ NTP induced the decrease more than other gases.

We also observed the inhibitory effect of NTP by various gases on OSCC cell invasion via the FAK and MMP system. FAK is a non-receptor tyrosine kinase whose expression increased in various invasive and metastatic cancer including OSCC.²⁸ FAK signalings are also correlated to tumor invasion during cancer metastasis by ECM degradation.²⁹ The connection of FAK and MMP-2/9 pathway has been shown previously;³⁰ FAK promotes cell migration via the process of combination with Src and subsequent paxillin phosphorylation.³¹ In cancer cells, the inhibition of FAK decreases MMP-9 secretion.³² Our present results are in agreement with earlier studies, since the cell migration was inhibited by suppression of FAK and paxillin signaling after NTP treatment with various gases, even though the most prominent results were induced by N₂ NTP.

In summary, N₂ NTP suppressed OSCC cell migration and invasion more strongly than the other two gases, and most potently reduced FAK, integrin β 3, and paxillin expression, which play key roles in tumor migration. N₂ NTP also significantly attenuated the activity of MMP-2/9, suggesting that N₂ might be more useful gas than Ar or He NTP for cancer treatment by NTP.

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