Hyperoxia sensitizes hypoxic HeLa cells to ionizing radiation by downregulating HIF-1α and VEGF expression

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Abstract. The current study investigated whether hyperoxia may reverse hypoxia-induced radioresistance (RR) in cervical cancer. Human HeLa cells exposed to hypoxic, normoxic or hyperoxic conditions were irradiated using X-rays. Cell proliferation and apoptosis were analyzed using MTT assays and flow cytometry. The expression levels of hypoxia-inducible factor-1α (HIF-1α), VEGF, VEGFRs, Akt and ERK were measured via western blotting and/or ELISA. The results demonstrated that hyperoxia stimulated HIF-1α and VEGF expression, and induced RR in HeLa cells. The administration of recombinant VEGF or the forced expression of VEGF promoted RR, whereas inactivating HIF-1α or blocking the VEGF-VEGFR interaction abrogated hypoxia-induced RR. Notably, hyperoxia decreased the level of hypoxia-stimulated HIF-1α and VEGF, and enhanced radiosensitivity in hypoxic HeLa cells. The results demonstrated that hyperoxia suppressed the hypoxia-activated Akt and ERK signaling pathways in HeLa cells. Therefore, a high O2 concentration may be considered as a radiotherapeutic sensitizer for hypoxic HeLa cells.

Introduction

Cervical cancer has been the second most common gynecological malignancy in women since the 1990s (1) and results in >300,000 deaths per year worldwide (2). Radiotherapy is the most frequently used therapeutic modality for cervical cancer (3); however, due to the occurrence of radioresistance (RR), the therapeutic efficacy of radiotherapy is far from satisfactory (4). Therefore, exploring effective and alternative methods to enhance radiosensitivity (RS) is crucial to the clinical radiotherapy of cervical cancer.

Intratumoral hypoxia is a typical characteristic of numerous invasive solid tumors (5). It has been proposed that hypoxic tumor cells achieve RR more easily compared with those in a normoxic environment, and therefore, tumor hypoxia has been associated with poor survival outcomes (6,7). Although the application of novel radiosensitizers, including Ro 90-7501, cisplatin (or nedaplatin) and curcumin, have enhanced the radiotherapy efficacy of cervical cancer (8-11), the toxic effects, such as acute hematological and gastrointestinal toxicity, induced by these chemicals are difficult to avoid.

Most tumors contain O2- and nutrient-deprived compartments (12). The sterilization of tumor cells under hypoxic conditions requires radiation doses that are three times higher than those for cells under normoxic conditions (13). Hyperbaric O2 therapy is an efficient method to cope with the phenomenon of hypoxia by enhancing the O2 load in tumor areas and enhance the response to ionizing radiation (IR) (14-16). Nordsmark et al (17) demonstrated that increased tumor partial O2 pressure enhanced IR response in mammary carcinoma. Pietrofesa et al (18) reported that exposure to the combination of IR + O2 increased DNA damage and cell death compared with the individual exposures to IR or O2 alone. However, how a high concentration of O2 pretreatment affects the efficacy of cervical cancer radiotherapy has not been fully elucidated.

Tumor cells in a hypoxic region adapt to low O2 tension conditions by activating survival factors and cell signaling pathways, such as hypoxia-inducible factor 1α (HIF-1α) and VEGF (19). HIF-1α is one of the most recognized transcription factors used by hypoxic cells in the harsh tumor microenvironment, and it activates >100 downstream genes required for tumor survival and progression, including VEGF, erythropoietin and c-MYC (20). The Akt and ERK signaling pathways are two major upstream regulators of HIF-1α. Previous studies have reported that there is an association between the Akt/ERK signaling pathways and HIF-1α/VEGF expression, which may affect the cellular reaction to radiation, O2 tension and chemical stimuli (21-23). However, the roles that HIF-1α, VEGF and the Akt/ERK signaling pathways serve in cervical cancer radiotherapy remain unclear.

The aim of present study was to investigate how hypoxia, normoxia and hyperoxia affect the efficacy of radiotherapy for cervical cancer and whether hyperoxia can reverse hypoxia-induced IR.

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Materials and methods

Cell line and culture. Human cervical cancer HeLa cells were purchased from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences and stored in the laboratory of Department of Obstetrics and Gynecology in the First Hospital of Jilin University. Cells were cultured in DMEM (Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (HyClone; Cytiva) and 1% antibiotics (penicillin/streptomycin; Invitrogen; Thermo Fisher Scientific, Inc.) in a humidified 37°C incubator with 5% CO₂.

Normoxia, hypoxia, reoxygenation and hyperoxia. For the normoxic culture (Normo), HeLa cells were maintained at 37°C in a humidified O₂ (20%), CO₂ (5%) and N₂ (75%) atmosphere (Thermo Fisher Scientific, Inc.). For the hypoxic culture (Hypo), cells were cultured at 37°C in a humidified O₂ (1%), CO₂ (5%) and N₂ (94%) incubator (Thermo Fisher Scientific, Inc.) for 12 h. For reoxygenation (Reoxy), cells were cultured under hypoxic conditions at 37°C for 12 h followed by normoxic conditions at 37°C for 2 h. For the hyperoxic culture (Hyper), cells were placed in a CO₂ (5%) and O₂ (95%) chamber (ProOX P360 O₂ controller and chamber; BioSpherix, Ltd.) at 37°C for 2 h (24). In order to investigate the effects of hypoxia, reoxygenation and hyperoxia on IR, HeLa cells were divided into the following treatment groups: i) IR-alone (IR); ii) hypoxia followed by IR (Hypo + IR); iii) hyperoxia followed by IR (Hyper + IR); iv) hypoxia followed by reoxygenation and IR (Hypo + Reoxy + IR); and v) hypoxia followed by hyperoxia and IR (Hypo + Hyper + IR).

IR. Monolayer HeLa cells were seeded into six-well plates (1x10⁵ cells per well) or 10-cm dishes (6x10⁵ cells per dish) to achieve 70% confluency and were then exposed to X-rays at a dose rate of 1 Gy/min in a X-RAD 320 (Precision X-Ray, Inc.). A moderate X-ray dose of 6 Gy was applied, according to previous studies (25,26). Following IR, cells were cultured for 24 h at 37°C in a humidified O₂ (20%), CO₂ (5%) and N₂ (75%) atmosphere until cell proliferation and apoptosis tests were conducted. For other experiments (e.g. western blotting), cells were harvested immediately. Control groups were treated similarly; however, these cells did not undergo IR.

Plasmid and cell transfection. Coding sequence of VEGF<sub>165</sub> was synthesized (Sangon Biotech Co., Ltd.) and cloned into pcDNA3.1 vector (Invitrogen; Thermo Fisher Scientific, Inc.) to form a pcDNA3.1-VEGF plasmid. For the forced expression of VEGF<sub>165</sub> (feVEGF) in HeLa cells, 2 µg plasmid DNA and blank control vectors were transfected using Lipofectamine<sup>®</sup> 3000 (Invitrogen), according to the manufacturer's protocol. Western blotting or IR (feVEGF + IR) were conducted at 48 h post-transfection.

VEGF/VEGFR stimulation and neutralization tests. For the VEGF stimulation test, 10 ng/ml recombinant human VEGF<sub>165</sub> (reVEGF, cat. no. ab9571; Abcam) (27) was added to cell culture media (37°C, 5% CO₂) at 2 h prior to IR (reVEGF + IR). For the ligand-receptor neutralization test, 25 µg/ml VEGF, 30 µg/ml VEGFR1 or 500 ng/ml VEGFR2 neutralization antibodies (cat. nos. AF293, AF321 and MAB3572; R&D Systems China Co., Ltd.) were added to cell culture media (37°C, 5% CO₂) at 2 h prior to IR, as previously described, and the manufacturer's protocol (28,29). Human IgG1 κ (cat. no. ab206200; Abcam) was used as the isotype control (IgG1 κ + Hypo + IR).

Inhibitors. To inhibit HIF-1α function, 100 nM echnomycin (30) (Merck KGaA) was added to cell culture media (37°C, 5% CO₂) 2 h prior to hypoxic exposure. To block the Akt and ERK signaling pathways, 50 µM LY294002 and 20 µM U0126 (Beyotime Institute of Biotechnology) were added to cell culture media (37°C, 5% CO₂) 2 h prior to IR as previously described (31,32).

ELISA. The level of secreted VEGF in the medium was quantified using a human VEGF Quantikine ELISA kit (cat. no. DVE00; R&D Systems, Inc.). According to the manufacturer's protocol, the supernatant of ≥1x10⁵ HeLa cells (or treated HeLa cells) were collected and centrifuged at 4°C and 1,000 x g for 15 min to remove debris and particulates. The absorbance at 450 nm (A<sub>450</sub>), which is proportional to VEGF concentration, was determined using an ELISA reader (Synergy HI Hybric Multi-Mode Reader; BioTek Instruments, Inc.).

VEGFR2 was quantified using a human VEGF R2/KDR Quantikine ELISA kit (cat. no. DVR200; R&D Systems, Inc.). According to the manufacturer's protocol, ≥1x10⁵ cells were washed 3 times in cold PBS and resuspended in 1 ml lysis buffer. The lysate was incubated at RT for 1 h with gentle agitation and centrifuged at 1,000 x g for 15 min at RT. A<sub>595</sub> was determined as aforementioned.

Cell proliferation inhibition assay. Cell proliferation was determined using a standard spectrophotometric MTT assay. HeLa cells were seeded at a density of 5,000 cells/well into a 96-well plate and underwent Hypo, Reoxy, Hyper and/or IR, as aforementioned. Subsequently, cells were cultured at 37°C for another 24 h in normal O₂ condition and incubated at 37°C for 4 h with MTT solution (5 mg/ml, Sigma-Aldrich; Merck KGaA). The cell supernatant was carefully aspirated, and the precipitate was dissolved using 200 µl DMSO (Sigma-Aldrich; Merck KGaA) at 37°C for 10 min. A<sub>590</sub> was determined as aforementioned. The cell proliferation inhibition rate (%) of each group was defined as follows: (A<sub>590</sub> of the experimental group-A<sub>590</sub> of the control group)/A<sub>590</sub> of the control group.

Apoptosis assay. Apoptosis was determined using an Annexin V-FITC Apoptosis Detection kit (cat. no. 556547; BD Biosciences). According to the manufacturer's protocol, 1x10⁵ HeLa cells were washed with cold PBS and centrifuged at RT and 200 x g for 5 min. The cell pellet was resuspended and incubated in 50 µl Annexin V solution containing 5 µl PI and 5 µl Annexin V-FITC at RT for 15 min. Data acquisition and analysis were performed using flow cytometry (FACScan; BD Biosciences; BD Biosciences) and FlowJo software (version 10.0; FlowJo LLC). Results for early and late apoptosis were combined as the total amount of apoptosis.
Western blotting. Total protein was extracted from HeLa or transfected HeLa cells using RIPA lysis buffer (Beyotime Institute of Biotechnology) supplemented with cocktail protease inhibitors [Roche Diagnostics (Shanghai) Co., Ltd.] and quantified using a Bradford dye binding assay kit (Beyotime Institute of Biotechnology) according to the manufacturer’s protocol. Each sample was loaded with 20 μg protein/lane on a 12% gel and separated via SDS-PAGE. Samples were electrophoretically transferred to a PVDF membrane (EMD Millipore), which was then blocked with 5% skimmed milk in PBS at RT for 1 h and incubated with the following primary antibodies against VEGF (1:1,500; cat. no. AF-293-SP; R&D Systems, Inc.), HIF-1α (1:1,000; cat. no. ab1; Abcam), ERK1/2 (1:1,000; cat. no. 4696; Cell Signaling Technology, Inc.), phosphorylated (p-)ERK1/2 (1:1,000; cat. no. 4370; Cell Signaling Technology, Inc.), Akt (1:1,000; cat. no. 4685; Cell Signaling Technology, Inc.), p-Akt (1:1,000; cat. no. 4060; Cell Signaling Technology, Inc.) and β-actin (1:1,500; cat. no. sc-47778; Santa Cruz Biotechnology, Inc.) at 37˚C for 1 h. Membranes were then incubated with goat anti-mouse, goat anti-rabbit or rabbit anti-goat (1:3,000; cat. nos. sc-2005, sc-2004 and sc-2768, respectively; Santa Cruz Biotechnology, Inc.) IgG peroxidase-conjugated secondary antibodies at 37˚C for 45 min. Immunoreactivity was visualized using an ECL detection kit (EMD Millipore). Densitometric analysis was performed using Quantity One software (version 4.6; Bio-Rad Laboratories, Inc.).

Statistical analysis. All experiments were repeated ≥3 times. Data were analyzed using SPSS software (version 17.0; SPSS, Inc.) and presented as the mean ± SD. Statistical analysis was performed using Student’s unpaired t-test or one-way ANOVA followed by Fisher’s Least Significant Difference, Bonferroni and Sidak post-hoc tests. P<0.05 was considered to indicate a statistically significant difference.

Results

Hyperoxic treatment enhances RS in hypoxic HeLa cells. The cellular inhibition rates were as follows: IR, 51.36%; Hypo + IR, 26.61%; Hyper + IR, 49.42%; Hypo + Reoxy + IR, 28.94%; and Hypo + Hyper + IR, 45.96% (Fig. 1). These data indicated that hypoxia promoted RR in HeLa cells and that hyperoxia sensitized the hypoxic HeLa cells to IR; however, reoxygenation did not sensitize cells to IR. There is a possibility that extending the reoxygenation time may achieve the same effect as the cells being exposed to hyperoxia for 2 h. However, this hypothesis requires further experimental verification.

Hyperoxia pretreatment significantly decreased the cellular apoptosis rates compared with the IR-alone (15.91% vs. 25.59%; Fig. 2). However, hypoxic cells which subsequently underwent hyperoxia exhibited a significant increase in RS and cellular apoptosis rate (27.26%) compared with cells which underwent reoxygenation (18.75%) or hypoxia-alone (15.91%).

Hyperoxia downregulates hypoxia-stimulated HIF-1α and VEGF expression. Hypoxia has been reported to stimulate VEGF expression, particularly in cancer cells (33). Western blotting showed that hypoxia treatment upregulated HIF-1α levels 2-fold and VEGF levels 2.3-fold compared with cells cultured in normal O2 (Fig. 3A and B). Reoxygenation decreased HIF-1α levels in hypoxic cells to a similar level as the untreated cells but did not affect VEGF levels (Fig. 3A and B). By contrast, hyperoxia decreased both the HIF-1α and VEGF levels in hypoxic cells (Fig. 3A and B). ELISA indicated that reoxygenation or hyperoxia decreased secretory VEGF levels in hypoxic cells (Fig. 3C).

VEGF enhances RR. VEGF has been reported to be involved in hypoxia-induced RR in HeLa cells (34). The current study treated HeLa cells with 10 ng/ml reVEGF or feVEGF to HeLa cells. Western blotting showed that VEGF levels increased 1.62-fold in HeLa transfectants (Fig. 4A). Cell apoptosis tests were conducted at 24 h post-IR; the apoptosis rates of the reVEGF and feVEGF treatment groups were 18.88% and 16.29%, respectively, compared with 27.16% in the untreated group (IR-alone) (Fig. 4B). These results suggested that reVEGF and feVEGF elevated the RR of HeLa cells.

Blocking the VEGF-VEGFR2 interaction decreases hypoxia-induced RR. VEGFRs are required for VEGF-based signal transfer (35). The current study used VEGF, VEGFR1 and VEGFR2 neutralization antibodies to verify whether the VEGF-VEGFR interaction is implicated in the hypoxia-induced RR. As presented in Fig. 5, treatment with VEGF and VEGFR2 neutralization antibodies significantly promoted IR-induced apoptosis (23.79 and 25.00%) compared with the Hypo + IR group (17.61%). However, administration of VEGFR1 neutralization antibodies did not significantly affect IR-induced apoptosis. These data indicated that VEGF may induce RR through interaction with VEGFR2, but not VEGFR1.

HIF-1α inactivation suppresses VEGF and VEGFR2 expression, and increases RS in hypoxic HeLa cells. Since the aforementioned results demonstrated that HIF-1α levels paralleled those of VEGF following hypoxic and hyperoxic treatments, it was hypothesized that HIF-1α may interact with VEGF and function collectively to induce
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RR in hypoxic HeLa cells. Therefore, the HIF-1α inhibitor echinomycin was added to the cell media prior to hypoxia treatment. The results demonstrated that the levels of VEGF (Fig. 6A) and VEGFR2 (Fig. 6B) in cells pretreated with echinomycin were significantly decreased compared with those in hypoxic cells. Furthermore, the results revealed that
the administration of echinomycin promoted IR-induced apoptosis in hypoxic HeLa cells (Fig. 6C). The cellular apoptosis rate in the Hypo + echinomycin + IR group increased by 1.7-fold compared with the Hypo + IR group (27.72% vs. 16.62%, respectively; Fig. 6D).

Hyperoxia enhances RS by suppressing the Akt and ERK signaling pathways. It has been reported that hyperactivation of Akt and ERK is associated with RR (36-38). The results of the current model detected the activation of Akt and ERK1/2. Western blotting results indicated that the levels of p-Akt (Fig. 7A) and p-ERK1/2 (Fig. 7B) were significantly increased in hypoxic HeLa cells compared with those in cells cultured in normal O\(_2\), whereas hyperoxia exposure significantly suppressed the phosphorylation of Akt and ERK1/2 induced by hypoxia. Furthermore, LY294002 (Fig. 7C) and U0126 (Fig. 7D) were used to block the PI3K/Akt and MAPK/ERK pathways, respectively, and the results demonstrated that hypoxia-induced RR was decreased and the IR-induced apoptosis was increased using the inhibitors.

Figure 4. Treatment with reVEGF or feVEGF enhances the radioresistance of HeLa cells. (A) Western blotting was performed to verify the forced expression of exogenous VEGF. (B) Flow cytometry results demonstrated that treatment with reVEGF or feVEGF decreased IR-induced apoptosis. Student’s t-test or one-way ANOVA followed by Fisher’s Least Significant Difference test. *P<0.05 and **P<0.01. reVEGF, recombinant VEGF; feVEGF, forced expression of VEGF; IR, ionizing radiation; normo, normoxia; CT, control.

Figure 5. Blocking the VEGF-VEGFR2 interaction decreases hypoxia-induced radioresistance. VEGF and VEGFR2 neutralization antibodies were added to the cell culture media to block the VEGF-VEGFR interaction. (A and B) Flow cytometry results indicated that administration of the VEGF neutralization antibody and VEGFR2 neutralization antibody promoted IR-induced apoptosis in hypoxic HeLa cells. However, administration of the VEGFR1 neutralization antibody did not affect IR-induced apoptosis. ANOVA followed by Bonferroni and Sidak post-hoc tests. **P<0.01. IR, ionizing radiation; Hypo, hypoxia.
Most, if not all, solid tumors exist in an anoxic microenvironment due to insufficient blood supply (39). To survive in hypoxic conditions, tumor cells gradually adapt to hypoxic stress through regulation of numerous genes, such as HIF-1α, VEGF, insulin-like growth factor α and transforming growth factor α, which are associated with tumor cell proliferation, migration and invasion. HIF-1α expression is upregulated by hypoxia through the stabilization of HIF-1α protein, which then translocates to the nucleus to activate the transcription of hypoxia-responsive genes (39). HIF-1α is known to regulate key genes involved in angiogenesis, glucose metabolism and cellular survival in the context of hypoxic conditions (39).

**Discussion**

Most, if not all, solid tumors exist in an anoxic microenvironment due to insufficient blood supply (39). To survive in hypoxic conditions, tumor cells gradually adapt to hypoxic stress through regulation of numerous genes, such as HIF-1α, VEGF, insulin-like growth factor α and transforming growth factor α, which are associated with tumor cell proliferation,
apoptosis, chemotherapy resistance and radiotherapy resistance (40).

Tumor hypoxia is a major contributing factor for the failure of anticancer therapies, including radiotherapy (7). It has been proposed that cells under hypoxic stress are more likely to generate RR due to the lack of O\(_2\) as a source of radiation-induced radicals and DNA damage (41). By contrast, increasing O\(_2\) pressure within the tumor can overcome hypoxia-induced RR (42). Pietrofesa et al (18) demonstrated that the combination of hypoxia and IR increased cell death and DNA damage compared with hypoxia or IR alone in mouse lung cells.

Mammalian cells respond to O\(_2\) deprivation by mobilizing multiple intracellular signal transduction pathways, including HIF family-dependent pathways (5). HIF-VEGF axis-mediated cell survival and angiogenesis are implicated in the radiation response in tumors. Fu et al (34) reported that hypoxic conditions enhance the RR of HeLa cells dependent on HIF-1\(\alpha\) by elevating VEGF expression and inhibiting p53 expression. Gorski et al (43) indicated that IR-induced VEGF expression may contribute to the protection of tumor blood vessels from cytotoxicity-mediated radiation; thereby, to tumor RR. The results of the current study confirmed that hypoxia induced RR in cervical cancer cells via the activation of HIF-1\(\alpha\) and VEGF. Administration of recombinant VEGF or introduction of exogenous VEGF increased RR in HeLa cells. By contrast, inactivation of a HIF-1\(\alpha\) or blockage of the VEGF-VEGFR interaction abrogated hypoxia-induced RR. According to the present results, it was hypothesized that tumor cells may secrete VEGF to build blood vessels and increase O\(_2\) and nutrients supply in order to survive IR-mediated cytotoxicity.

VEGFR1 and VEGFR2 have highly homologous structures; however, their biological functions differ (44). VEGFR2 is implicated in pathological and physiological angiogenesis, whereas VEGFR1 acts as a decoy receptor that limits VEGFR2 activation (45,46). Ding et al (47) reported that silencing of HIF-1\(\alpha\) promoted cell apoptosis and inhibited cell invasion by downregulating VEGF and VEGFR2 expression in thyroid cancer. The results of the current study demonstrated that VEGF may function as an IR-resistant factor through interaction with VEGFR2. Additionally, inhibition of HIF-1\(\alpha\) may abrogate hypoxia-induced RR through the downregulation of VEGF and VEGFR2 expression. The present findings indicated that the HIF-1\(\alpha\)-VEGF axis may serve an important role in regulating the response of hypoxic cervical cancer cells to IR.

The results of the present study demonstrated that hypoxia treatment significantly sensitized hypoxic HeLa cells to IR and promoted cellular apoptosis. To gain further insight into the molecular mechanism contributing to this phenomenon, the activation of the Akt and ERK signaling pathways was detected, and the results revealed that hypoxia treatment increased the protein expression levels of p-Akt and p-ERK, whereas hypoxia abrogated hypoxia-activated p-Akt and p-ERK. Additionally, the administration of Akt and ERK pathway inhibitors significantly decreased hypoxia-induced RR. Gupta et al (48) demonstrated that VEGF significantly enhanced the survival of vein endothelial cells in radiotherapy via the activation of MEK and ERK. Additionally, Chen et al (49) reported that antrocin synergistically induced cell apoptosis and inhibited cell proliferation in radioresistant prostate cancer cells by suppressing the Akt and ERK signaling pathways. The current study indicated that hypoxia may function as a radiosensitizer by suppressing the hypoxia-induced hyperactivation of the Akt and ERK signaling pathways. However, to validate IR-induced cellular apoptosis, detection of the changes of additional apoptosis markers, including p53, poly (ADP-ribose) polymerase, the caspase cascade and the Bcl-2 family (50-52), should be investigated in future work.

In our previous work, different tumor cell lines were investigated; these cell lines exhibited different sensitivity to IR and O\(_2\) (data not shown). Therefore, there may be a tumor type-specific association among RR, the HIF-1\(\alpha\)-VEGF axis and hypoxic sensitization. The present study only reported the data from HeLa cells, as HeLa cells are the most commonly used cell model for the research of cervical cancer. However, to obtain a more comprehensive and solid conclusion, further experiments using more cervical cancer cell lines and in vivo assays are required.

In conclusion, the current investigation demonstrated that hypoxia may be considered as a radiotherapeutic sensitizer for hypoxic HeLa cells.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JW and DD wrote the manuscript and performed hypoxia and hypoxia culturing, cell irradiation, cell proliferation and apoptosis detection. YF contributed to the experimental design. FC and JZ performed plasmid construction experiments. HJ and JL performed the ELISA and western blotting experiments. HW performed the antibody neutralization tests and contributed to statistical analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.
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