Osthole suppresses the proliferation and induces apoptosis via inhibiting the PI3K/AKT signaling pathway of endometrial cancer JEC cells

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Received March 7, 2020; Accepted March 19, 2021

DOI: 10.3892/etm.2021.10605

Abstract. Osthole, a natural product extracted mainly from fruits of *Fructus Cnidii*, possesses multiple pharmacological functions, including anti-inflammatory, anti-convulsant and anticancer effects. However, the effects of osthole in endometrial cancer (EC) is not fully understood. In the present study, EC cell lines, including JEC, KLE and Ishikawa cells and normal human cervical epithelial cells (HcerEpic) were applied to detect the anticancer effect of osthole. The present study demonstrated that osthole inhibited the proliferation of JEC, KLE and Ishikawa cells, but had no cytotoxic effect on HcerEpic. Furthermore, treatment of osthole induced JEC cell apoptosis, while osthole promoted the release of pro-apoptotic proteins, Bax and activated the cleaved caspase-3, caspase-9 and PARP. Additionally, osthole significantly increased the expression of PETN and decreased the phosphorylated form of PI3K and AKT in a concentration-dependent manner. Furthermore, osthole treatment suppressed the JEC tumor cell growth in a nude mouse xenograft model *in vivo*, and neither renal toxicity nor hepatotoxicity was induced by the indicated concentration. Taken together, the results of the present study suggested that osthole may be a novel and potential therapeutic agent of EC.

Introduction

Endometrial cancer (EC) is one of the most common gynecological cancer types, and the incidence has accounted for the majority of all gynecological malignancies in America and European countries (1,2). The risk factors of EC include obesity, hypertension, diabetes, menopause disorder and

long-term stimulation of estrogen (3,4). In recent years, with the rapid economic development of China, the incidence of uterine cancer was significantly increased due to changes in people's living habits and diet structure. Furthermore, it has become a serious threat to the health of women, a severe social issue and has a huge financial burden on the country (5,6). The first choice for the treatment of EC is hysterectomy and bilateral salpingo-oophorectomy, while multiple studies have demonstrated that laparoscopic surgery possesses more advantages than laparotomy, including lower degree of blood loss, fewer wound complications and shorter hospital stays (7,8). However, there is no difference in the 5-year overall survival rate between robotic surgery and laparotomy. Furthermore, it was reported that in patients with stage I intermediate-risk EC, robotic surgery was associated with a higher recurrence rate than laparotomy (9-11). The majority of patients with advanced stage EC or for those at high risk of recurrence require comprehensive therapy, including drug treatment, adjuvant chemotherapy and radiotherapy (12,13). However, the management of endometrial cancer remains a clinical challenge (14-16). Therefore, it is essential to enrich the strategies of prevention and treatment for endometrial carcinogenesis. More therapeutic methods and effective drugs require investigation. The present study focused on alternate treatment options, including phytochemicals such as natural herb compound, which has few side effects and a higher specificity.

Osthole is a natural compound mainly extracted from *Fructus Cnidii*, which has been widely used in traditional Chinese medicine. It possesses a variety of pharmacological properties, including anti-inflammatory, anti-angiogenic, stimulates bone formation and ameliorates cartilage degradation (17-20). A large number of studies have proven that osthole has an effective anticancer effect in various tissues, including gallbladder cancer (21), ovarian cancer (22), head and neck carcinoma (23), pancreatic cancer (24), esophageal squamous cell carcinoma (25) and breast cancer (26,27). However, the effects of osthole on EC have not been fully investigated.

The PI3K/AKT pathway is a major survival pathway in numerous cancer types (28). It serves a key role in regulating cell growth, migration, apoptosis and survival in EC (29). Activation of the PI3K/AKT pathway has been well studied in the tumorigenesis and progression of EC (30). It has been

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Key words: apoptosis, osthole, endometrial cancer, PI3K/AKT

proven that a decrease in PTEN may lead to activation of the PI3K pathway, and loss of PTEN protein may increase phosphorylation of AKT, which in turn influences its target genes to increase cell growth and decrease apoptosis (31). The present study aimed to investigate the cytotoxic effect of osthole in EC cell lines (JEC, KLE, and Ishikawa) and its underlying mechanisms involving the PI3K/AKT pathway.

Materials and methods

Cell culture. Human endometrial cancer KLE and Ishikawa cell lines were purchased from American Type Culture Collection. The human EC JEC cell line and the normal human cervical epithelial HcerEpic cell line were purchased from Shanghai YaJi Biotechnology Co., Ltd. Cells were cultured in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Inc.), supplemented with 10% FBS (Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Thermo Fisher Scientific, Inc.). All cells were cultured at 37°C and 5% CO₂ atmosphere.

Cell proliferation assay. The effect of osthole on the viability of EC cells was measured using a CCK-8 kit. CCK-8 kit (kit no. CK04) was purchased from Dojindo Molecular Technologies, Inc. and Osthole powder (O9265) was purchased from Sigma-Aldrich (Merck KGaA). In brief, EC cells (JEC, KLE and Ishikawa) and normal human cervical epithelial cells (HcerEpic) were seeded onto 96-well plates overnight. On the second day, cells were treated with various doses of osthole (0, 25, 50, 100 and 200 μ m) for different amounts of time (24, 48 and 72 h) at 37°C. At the end of the experiment, CCK-8 (10 μ l) was added and the cells were incubated for another 4 h at room temperature. Finally, the absorbance was measured at 450 nm (OD 450).

Cell apoptosis analysis. Apoptosis analysis was performed by flow cytometry. An Annexin V-FITC/PI apoptosis detection kit (cat. no. 556547) was purchased from BD Biosciences. EC JEC cells were treated with paclitaxel (10 μ m; Sigma-Aldrich; Merck KGaA) or different doses of osthole (25, 50 and 100 μ m; Sigma-Aldrich; Merck KGaA) for 48 h at 37°C. A total of 1x10⁵ cells were then collected and washed twice with PBS. Subsequently, cells were resuspended in 100 μ l binding buffer containing 5 μ l Annexin V and 5 μ l PI. Cells were kept at room temperature in the dark for 15 min, and a total of 400 μ l 1X binding buffer was subsequently added and flow cytometric analysis was performed (FACSCalibur™; BD Biosciences). Data were analyzed by Flow Jo v10 (BD Biosciences).

Western blotting. JEC cells were exposed to different doses (0, 25, 50 and 100 μ m) of osthole for 48 h at 37°C. Then cells were extracted by RIPA buffer [50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 0.5% Nonidet P-40, 1 mM NaF and 1 mM PMSF Protease Inhibitor Cocktail 0.02% (v/v; pH 7.4)]. The concentration of protein was analyzed using a BCA Protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). The protein samples (50 μ g per lane) were loaded onto 8 or 10% SDS-polyacrylamide gel, prior to being transferred to PVDF membranes. Membranes were blocked with 5% skimmed milk at 37°C for 2 h and then incubated with specific

primary antibodies at 4°C overnight. The primary antibodies: phosphorylated (p)-AKT (1:1,000; cat. no. 9271S); AKT (1:1,000; cat. no. 4685S); p-mTOR (1:1,000; cat. no. 2971S); and mTOR (1:1,000; cat. no. 2983S) were purchased from Cell Signaling Technology, Inc. The following antibodies: Bcl-2 (1:1,000; cat. no. ab196495); PTEN (1:1,000; cat. no. ab170941); PI3K (1:1,000; cat. no. ab191606); p-PI3K (1:1,000; cat. no. ab182651); cleaved-caspase3 (1:1,000; cat. no. ab2302); cleaved-caspase-9 (1:1,000; cat. no. ab2324); and Bax (1:1,000; cat. no. ab32503) were purchased from Abcam. A GAPDH antibody (1:1,000; cat. no. sc-47724) was purchased from Santa Cruz Biotechnology, Inc. Following washing twice with TBS with 0.1% Tween-20, membranes were exposed to the following HRP-conjugated secondary antibodies for 2 h at 37°C: Anti-rabbit (1:5,000; cat. no. ab97051; Abcam) and anti-mouse (1:5,000; cat. no. ab97023; Abcam). The signal was visualized using chemiluminescent detection reagent (cat. no. ab133406; Abcam).

ELISA assay. AKT ELISA kit (cat. no. ab126433) and PI3K ELISA kit (cat. no. ab207484) were purchased from Abcam. In brief, JEC cells were exposed to different doses (0, 25, 50 and 100 μ m) of osthole at 37°C for 48 h. Next, cells were collected and solubilized in cell lysis buffer taken from the aforementioned ELISA kit (Abcam), prior to lysates being resuspended and incubated at 4°C for 30 min. Following centrifugation (10,000 x g for 10 min at 4°C), 100 μ l of each supernatant was collected in appropriate wells and incubated overnight at 4°C. A total of 100 μ l antibody taken from the aforementioned ELISA kit (Abcam) was diluted 55-fold with 1X assay diluent according to the manufacturer's protocol, and added to each well for 1 h at 37°C after washing with wash buffer. Subsequently, 100 μ l HRP-conjugated anti-rabbit IgG [diluted 500-fold with 1X assay diluent taken from the aforementioned ELISA kit (Abcam), according to manufacturer's protocol] was added to each well for 1 h at 37°C. The solution was discarded and the washing was repeated. Next, 100 μ l 3,3',5,5'-tetramethylbenzidine one-step substrate reagent taken from the aforementioned ELISA kit (Abcam) was added, followed by 50 μ l stop solution being added to each well. The optical density was immediately measured at 450 nm.

Caspase-9 activity assay. The Caspase-Glo® 9 assay kit (kit no. G8211) was purchased from Promega Corporation. In brief, JEC cells were plated into 96-well plates and cultured overnight. Next, cells were exposed to different doses of osthole (0, 25, 50, 100 and 200 μ m) for different time periods (24, 48 and 72 h) at 37°C. The 96-well plates containing cells were removed from the incubator and 100 μ l Caspase-Glo 9 Reagent was added to each well. Following gently mixing contents of wells using a plate shaker at 40 x g for 30 sec at 4°C, the plate was incubated at 37°C for another 1 h. The luminescence was measured using a luminometer.

In vivo tumorigenicity assays. Female nude mice aged 5 weeks (weight, 19–21 g) were purchased from SPF (Beijing) Biotechnology Co., Ltd. In brief, suspension of JEC cells (2x10⁶) was subcutaneously injected into the dorsal of nude mice. A total of 12 mice were used in the experiment, and two of them were excluded because they did not bear evident tumors.

Figure 1. Osthole suppresses the growth of JEC, KLE and Ishikawa cells *in vitro*. JEC, KLE, Ishikawa and HcerEpic cells were incubated with 0, 25, 50, 100 or 200 μ M osthole for 24, 48 or 72 h. The values represent the mean $\pm$ standard error of the mean of three independent experiments. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$.

After 7 days, the mice bearing evident tumors were randomly distributed into two groups ($n=5$ each). The behavior and growth of tumors among the mice were observed daily. Each mouse was treated with either fresh medium for the control group or 20 mg/kg of osthole for the treatment groups through intraperitoneal injection every other day. The indoor temperature was controlled at 20–25°C, the humidity was controlled at 30–60%, the light/dark cycle was 12 h and free access to food and water. Animal waste was regularly removed and fresh air was regularly introduced. Tumor volume was calculated as $V = \text{length} \times \text{width}^2 / 2$. The maximum tumor volume was 982.8 mm³, and no multiple tumors occurred. All procedures were performed in accordance with the Institutional Animal Care and Use of the 980th Hospital of the Joint Logistic Support Force of the Chinese People's Liberation Army.

Evaluation of biochemical parameters. To assess changes in hepatic and renal function following *in vivo* experiment, blood samples were collected from the right retroorbital plexus of anesthetized mice two days prior to the animals being sacrificed. Anesthesia was induced by 50 mg/kg intraperitoneal injection of 1% pentobarbital sodium resolved in PBS. The levels of serum alanine transaminase, creatinine and blood urea nitrogen were used to assess changes in liver and renal function. These biochemical parameters were analyzed using the Roche Hitachi 911 Chemistry Analyzer (Roche Diagnostics GmbH) as described previously (32).

Anesthetics and euthanasia. At the end of the *in vivo* experiment, blood samples were collected from the right

retroorbital plexus of anesthetized mice. Anesthesia was induced by 50 mg/kg intraperitoneal injection of 1% pentobarbital sodium resolved in PBS. Additionally, the animals were euthanized by carbon dioxide anesthesia after 2 days. In brief, the animals were placed in the euthanasia box and then 100% CO₂ was introduced. The filling rate was ~10–30% CO₂ per min of the chamber volume. After the animals stopped breathing, which was observed by minimal chest undulation and the eyeballs turning white, CO₂ flow was maintained for at least 1 min, prior to the death of the animals being declared.

Statistical analysis. All experiments were conducted at least three times, and all values are provided as the means $\pm$ standard error of the mean of three independent experiments. Comparisons between two groups were analyzed using a Student's *t*-test. A one-way analysis of variance, followed by Tukey's post hoc test was used to analyze differences among multiple groups. GraphPad Prism 6.0 software was used to perform calculations, and $P < 0.05$ was considered to indicate a statistically significant difference (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Results

Osthole suppresses the growth of JEC, KLE and Ishikawa cells *in vitro*. Human endometrial cancer cell lines (JEC, KLE and Ishikawa) and a normal human cervical epithelial cells (HcerEpic) were exposed to different doses of osthole for 24, 48 or 72 h in order to determine the effects of osthole on cell

Figure 2. Osthole provokes apoptosis in JEC cells. (A) JEC cells were incubated with paclitaxel or different doses of osthole for 48 h, prior to the percentage of apoptosis cells being analyzed by flow cytometry. (B) The number of apoptotic cells accounts for the total cells in each group after 48 h treatment. The values represent the mean $\pm$ standard error of the mean of three independent experiments. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$

growth. As shown in Fig. 1A, none of these treatments with osthole revealed significant toxicity on HcerEpic cells, except the high dose (200 μM osthole) at a long exposure time (48 and 72 h), which indicated that the appropriate dose of osthole had little effect on normal human cervical epithelial cell. Furthermore, it was found that osthole exposure suppressed the growth of all three cancer cell lines in a dose- and time-dependent manner, and osthole demonstrated the most toxicity on JEC cells (Fig. 1B-D). Therefore, following these experiments, JEC cells were selected for subsequent research.

Osthole provokes apoptosis in JEC cells. To further investigate the anticancer potential of osthole, cell apoptosis of

JEC cells was measured by flow cytometry. Following being treated with paclitaxel or a different dose of osthole, JEC cells were collected and handled for flow cytometry detection. As shown in Fig. 2, the proportion of apoptotic JEC cells increased gradually while the concentration of osthole increased. Specifically, the proportion of apoptotic cells in the positive control group (10 μM paclitaxel group) was $48.1 \pm 6.4\%$ and that in the negative control group was $2.0 \pm 0.9\%$, while the number increased to $7.4 \pm 0.9\%$ in the 25 μM osthole treatment group, $28.2 \pm 4.2\%$ in the 50 μM group and $42.9 \pm 2.2\%$ in 100 μM group. These results suggested that osthole induced apoptotic cell death in JEC cells.

Figure 3. Osthole induces activation of caspase-9 in JEC cells. Fold-changes of caspase-9 activity compared with control JEC cells treated with osthole for 24, 48 or 72 h. The values represent the mean $\pm$ standard error of the mean of three independent experiments. * $P<0.05$, ** $P<0.005$.

Osthole induces activation of caspase-9 in JEC cells. The activation of caspase-9 is a critical process in the occurrence of apoptosis. The present study further investigated the effects of osthole on caspase-9 activities in JEC cells. As shown in Fig. 3, the exposure of osthole activated caspase-9 in a time- and dose-dependent manner. It was found that four different concentrations of osthole showed no obvious activation effect after 24 h treatment. However, caspase-9 activity was increased significantly following 48 h exposure to osthole, including low-dose osthole (25 μ M). These results implied that osthole treatment may induce the activation of caspase-9; therefore, the anticancer effect mediated by osthole on JEC cells is relevant to caspase-involved apoptotic pathways.

Osthole exhibits anticancer effect of JEC cells through inhibiting the PI3K/AKT signaling pathway. To gain a deep understanding of the underlying regulatory mechanisms involved in the anticancer effect of osthole, JEC cells treated with different concentrations of osthole were collected and Western blot analysis was performed. Annexin V-FITC/PI staining revealed that osthole provokes apoptosis of JEC; therefore, the expression of several apoptotic markers was investigated. As shown in Fig. 4A, expression of cleaved caspase-3, cleaved caspase-9, cleaved-PARP and Bax were increased by osthole treatment, particularly in the 100 μ M group, while protein expression of Bcl-2 was decreased in a concentration-dependent manner. The PI3K/AKT signaling pathway serves a key role in tumor metastasis, and activation of the PI3K/AKT pathway was associated with protection of cells from apoptosis. The present study investigated whether osthole inhibits PI3K/AKT activity and consequently leads to apoptosis of JEC cells. As shown in Fig. 4, the expression of PTEN increased following cells being treated with osthole, while the phosphorylated PI3K and phosphorylated AKT were significantly decreased. Furthermore, to better quantify the size of the effect, an ELISA-based system was used to determine the ratio of phospho-AKT/AKT and phospho-PI3K/PI3K, and a decrease in AKT and PI3K phosphorylation was observed in osthole-treated JEC cells, particularly in the 100 μ M group (Fig. 4C). These results indicated that osthole may induce

apoptosis of JEC cells via downregulation of the PI3K/AKT signaling pathway.

Osthole inhibits the growth of JEC xenografts in nude mice. To further investigate the tumor-suppressing potential of osthole, a model for tumorigenicity of JEC cells in nude mice was established. Each mouse bearing JEC cell xenografts was treated with either fresh medium for the control group or 20 mg/kg osthole for the treatment groups through intraperitoneal injection. As shown in Fig. 5, the tumor volume decreased significantly from day 16 after treatment with osthole, compared with the control group. Furthermore, osthole treatment had little effect on the body weight of the mice. Additionally, there was no significant differences in serum alanine transaminase, creatinine and blood urea nitrogen between mice treated with fresh medium or osthole, indicating that neither renal toxicity nor hepatotoxicity was induced by the indicated concentration of osthole *in vivo*.

Discussion

Even though there have been great improvements in surgical equipment and new anticancer drugs, the outcome of patients with EC has not notably improved over recent decades (33,34). There are an estimated 319,500 patients diagnosed with EC annually, which accounts for >76,000 deaths each year (35). Furthermore, patients with EC, particularly in the late stages, also require adjuvant therapy to improve progression-free and overall survival rates (36-39). However, acquired resistance to current chemotherapeutic drugs has greatly impaired the successful treatment of EC. Herbal medications have attracted the interest of clinicians recently due to its safety, limited side-effect and effectiveness.

Osthole has been reported to exert proliferation inhibiting effects and to induce the apoptosis of Ishikawa and KLE cells (40). The present study found that, compared with Ishikawa and KLE cells, EC JEC cells were more sensitive to osthole. Furthermore, osthole exhibited significant cytotoxicity in all three EC cell lines in a concentration and time-dependent manner. All these types of treatment with osthole revealed no significant toxicity on HcerEpic cells, except at a high dose (200 μ M osthole) for a long exposure time (48 and 72 h), indicating that the appropriate dose of osthole was safe to normal human cervical epithelial cells. The results obtained from the assays used in the present study (caspase-9 activity, apoptosis analysis and western blotting) also proved that osthole improved JEC cell apoptosis through a caspase-dependent pathway. After JEC cells were treated with osthole, the percentage of apoptotic cells increased significantly, particularly in the 100 μ M group. Anti-apoptotic and pro-apoptotic proteins of the Bcl-2 family are key regulators of apoptosis (41-43). The present study reported that expression of pro-apoptotic proteins, Bax, cleaved-caspase-3, -caspase-9 and -PARP were increased while expression of the anti-apoptotic protein, Bcl-2, was decreased. Additionally, the data indicated that caspase-9 activity was increased significantly after 48 h exposure of osthole, including low-dose osthole (25 μ M). Furthermore, it was noted that regardless of duration, caspase-9 activation in the 100 μ M osthole treatment group was higher than that in the 200 μ M group, indicating

Figure 4. Osthole exhibits anticancer effects on JEC cells through inhibiting the PI3K/AKT signaling pathway. (A) JEC cells were treated with different doses of osthole for 48 h and the expression of cleaved-caspase3, -caspase9, -PARP, Bcl-2, Bax, p-PI3K, PI3K, p-AKT and AKT were detected by Western blotting. (B) Quantitative results of indicated proteins, which were presented as the fold-change of the protein expression in the control. (C) Phospho-AKT/AKT and phospho-PI3K/PI3K were determined using ELISA. The values represent the mean $\pm$ standard error of the mean of three independent experiments. * $P < 0.05$, ** $P < 0.005$.

Figure 5. Osthole inhibits the growth of JEC xenografts in nude mice. (A) Tumor volume of mice carrying xenografts of the two groups were measured every two days. (B) The body weight of each group of mice was measured every six days. (C-E) Serum concentrations of indicated biochemical markers were measured after the end of experiment. The results showed that there was no significant difference in serum ALT, creatinine or BUN. * $P < 0.05$, ** $P < 0.005$. ALT, alanine transaminase; BUN, blood urea nitrogen.

that a high concentration of osthole may induce types of cell death other than apoptosis. The results implied that osthole treatment may induce the activation of caspase-9; therefore, the anticancer effect mediated by osthole on JEC cells is associated with the caspase-involved apoptotic pathway.

Numerous studies have proven that the PI3K/AKT signal pathway is associated with the progression of EC, and AKT activation may affect numerous pathways, including cell proliferation, migration and apoptosis (44-46). Therefore, this pathway was selected for further study, and it was found that, with the increased expression of PTEN, the expression of p-PI3K and p-AKT was decreased following exposure to osthole, suggesting that this pathway is associated with the anticancer effect of osthole in JEC cells.

Last but not least, the *in vivo* effect of osthole in EC cells was investigated. The results demonstrated that tumor volume had decreased significantly since day 16 after treatment with osthole, compared with the control group, while osthole treatment had little effect on body weight. Furthermore, there was no significant difference in serum alanine transaminase, creatinine and blood urea nitrogen between mice treated with fresh medium or osthole. Further *in vivo* studies are required, for example, combined use of osthole with chemotherapeutic drugs, to elucidate its exact mechanism and clinical therapeutic potential.

In conclusion, the present study investigated the anticancer effects of osthole in endometrial cancer cell lines. It was

found that osthole within a certain concentration range may inhibit cell proliferation in all three EC cells, but exhibited no significant cytotoxic effect on HcerEpic. Furthermore, flow cytometry revealed that treatment of osthole significantly increased JEC cell apoptosis, while the expression of pro-apoptotic proteins, Bax and cleaved caspase-3, caspase-9 and PARP, was increased. Additionally, osthole significantly increased the expression of PTEN, and decreased the activation of phosphorylation PI3K and AKT, suggesting that the PI3K/AKT signaling pathway was involved in this process. Furthermore, osthole treatment suppressed the growth of JEC tumor cells in a nude mouse xenograft model *in vivo*, without obvious renal toxicity or hepatotoxicity.

The present study aimed to contribute toward an improved understanding of the role of osthole in EC and it may be a safe and effective therapeutic agent for EC. However, whether osthole may induce types of cell death other than apoptosis, including autophagy or pyroptosis, remain unknown. The present study did not investigate target genes other than PTEN and the PI3K/AKT signaling pathway in EC, and whether knockdown of PTEN may reverse the antitumor effect of osthole has not been elucidated. Further experiments are required to verify the anticancer effect of osthole in EC.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Hebei Province Key Project of Medical Science Research (grant no. -20160005).

Availability of data and materials

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

Authors' contributions

LL and LS designed the study and carried out the experiments. LL and YW performed the analysis. BY participated in the design of the study. LL and YW confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

All procedures were performed in accordance with the Institutional Animal Care and Use of the 980th Hospital of the Joint Logistic Support Force of the Chinese People's Liberation Army.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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