Cell. Mol. Biol. (ISSN: 1165-158X)

Journal Homepage: www.cellmolbiol.org

Cellular and Molecular Biology

Original Article

Effect of ethyl pyruvate on human esophageal squamous cell carcinoma transplanted tumors in nude mice

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Article Info

Article history:

Received: April 07, 2024
Accepted: March 15, 2024
Published: July 31, 2024

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Abstract

The objective of this study was to investigate the impact of ethyl pyruvate (EP), an HMGB1 inhibitor, on ESCC cells both in vitro and in vivo. The viability of ESCC cells was assessed using the MTT method to evaluate the correlation between EP and cell viability. A scratch test was used to investigate the relationship between EP and cell migration and invasion. The effects of EP on tumor growth and survival in cancerous nude mice were examined using a tumor formation model. Immunohistochemical staining was performed to evaluate the expression levels of HMGB1, TLR4, and MyD88 in tumor tissues. EP, an anti-HMGB1 inhibitor, inhibited ESCC cell proliferation and metastasis in vitro and in vivo. Furthermore, compared with the control treatment, EP improved the activity, diet, and drinking behaviour of nude mice; inhibited tumour growth; and led to lower protein expression levels of HMGB1, TLR4, and MyD88. EP has the potential to regulate the HMGB1/TLR4-MyD88 signaling pathway, thereby inhibiting the proliferation and metastasis of ESCC, suppressing tumor growth, improving quality of life, and serving as an effective drug for ESCC treatment.

Keywords: Esophageal squamous cell carcinoma, Ethyl pyruvate, HMGB1, TLR4, MyD88.

1. Introduction

High mobility Box B1 (HMGB1) is a nonhistone nuclear transcription factor that mediates signaling pathways such as RAGE and TLR signaling pathways, promoting the expression of extracellular and extracellular inflammatory factors and participating in the pathogenesis and development of various diseases [1]. Numerous studies have established a close relationship between HMGB1 and esophageal squamous cell carcinoma (ESCC). Matsubara et al. reported that upregulating HMGB1 can enhance the phosphorylation of the ERK1/2 and NF-kBp65 proteins, thereby promoting the migration and invasion ability of ESCC cells [2]. Dong et al. demonstrated that HMGB1 overexpression may facilitate the malignant phenotype of ESCC cells and promote radioresistance by reducing G0/G1 arrest [3]. Li et al. reported that the coexpression of HMGB1 and RAGE was associated with the TNM stage, lymphatic infiltration, and venous infiltration of ESCC patients. They also identified venous infiltration, HMGB1 expression, and the coexpression of HMGB1 and RAGE as independent prognostic factors for ESCC [4]. Our previous in vitro cell experiments confirmed that the HMGB1-mediated TLR4-MyD88 signaling pathway positively regulates the proliferation, invasion, and metastasis of ESCC cells [3]. Therefore, blocking the HMGB1/TLR4-MyD88 signaling pathway may be a novel strategy for the prevention and treatment of ESCC.

Ethyl pyruvate (EP) is a recognized HMGB1 antagonist that modulates every step of HMGB1 biology [5]. It has anti-inflammatory, antioxidant, immunomodulatory, and neuroprotective effects [6, 7]. In recent years, EP has been demonstrated to inhibit the growth, invasion, and migration of tumor cells, including gallbladder cancer, liver cancer, and non-small cell lung cancer, and diffuse large B-cell lymphoma cells, by regulating the downstream signaling pathways of HMGB1 membrane receptors [8-11]. However, whether EP has potential therapeutic value in ESCC remains unclear. In this study, we investigated the regulatory effect of EP on the HMGB1/TLR4-MyD88 signaling pathway by treating ESCC cells in vitro and ob-

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Doi: http://dx.doi.org/10.14715/cmb/2024.70.7.30

serving tumor formation in nude mice.

2. Materials and methods

2.1. Experimental Materials

2.1.1. Experimental cells and animals

The human oesophageal squamous cell line ECAl09 used in this study was purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences. Twenty BALB/c-nu/nu mice were purchased from Shanghai Slack Experimental Animal Co., Ltd. ECAl09 cells in the logarithmic growth phase were digested with 0.25% trypsin to adjust the cell suspension to a concentration of 1.0×107/ml and injected subcutaneously into the right axil of nude mice at an injection dose of 0.2 ml/mouse. Twenty nude mice were bred in the Animal Laboratory of Lianyungang Hospital affiliated with Xuzhou Medical University. The room temperature was maintained at 25°C, and the humidity was 50 ± 10%. During the rearing period, the nude mice had free access to water and food. After feeding for 1 week, 10 nude mice with improved tumor uniformity were selected for inclusion in the study, and the tumor volume was maintained at 148±5 mm3. This study was approved by the Ethics Committee of Lianyungang Hospital affiliated with Xuzhou Medical University.

2.1.2. Experimental instruments and equipment

A light microscope [Osparin, Japan, model: CX41]; a microplate reader [Nanjing Baocheng Biotechnology Co., Ltd., Nanjing, China, model: 1681130-4A]; a cell incubator [Thermo Scientific, Waltham, MA, USA, model: 8000]; a low-speed centrifuge [Shanghai Luxiangyi Laboratory Instrument Co., Ltd., Shanghai, China, model: TDZ4B-WS]; an inverted photomicroscope [Leica, Germany, model: DMI3000B]; a high-resolution optical microscope [NIKON, Tokyo, Japan, model: Eclipse ci]; and a system [NIKON Corporation, Tokyo, Japan, model: DS-FI2].

2.1.3. Experimental reagents

EP (Sigma Corporation, St. Louis, MO, USA), 4% paraformaldehyde fixative solution (Beijing Suolaibao Technology Co., Ltd., Beijing, China), RPMI 1640 medium and PBS (HyClone Corporation, South Logan, UT, USA), trypsin-EDTA solution (Shanghai Yuanpei Biotechnology Co., Ltd., Shanghai, China), penicillin–streptomycin (Shanghai Sangon Bioengineering Co., Ltd., Shanghai, China), foetal bovine serum (GIBCO, USA), and MTT (Shanghai Beyuntian Biotechnology Co., Ltd., Shanghai, China) were used.

2.2. Experimental methods

2.2.1. Cell culture

Under sterile conditions, the human esophageal cancer ECAl09 cell suspension was injected into a centrifuge tube, after which 5 mL of RPMI 1640 culture solution was added, after which the mixture was centrifuged. The supernatant was discarded, and the culture medium was added to repeat the centrifugation once. Five milliliters of RPMI 1640 culture medium containing 10% fetal calf serum was added, and the cells were transferred to sterile culture flasks and incubated at 37°C in a 5% CO_2 cell incubator overnight, after which the culture medium was changed the next day. When the cells grew well and covered 90% of the bottom of the flask, the supernatant was discarded, the cells were washed twice with 2 ml of PBS, and the reaction was terminated by adding 0.25% trypsin. The digestion solution was discarded, the cultures were subcultured in 3-4 ml of RPMI 1640 culture solution by pipetting down to mix evenly, and the cultures were passaged at a ratio of 1:2-3 and placed in a 37°C, 5% CO_2 incubator. One sterile culture bottle was passed for 2-3 days.

2.2.2. MTT experiment

When the cells were in the logarithmic growth phase, the cell density was adjusted to 5×10^4 cells/ml, the cells were transferred to 96-well cell culture plates, and 150 µl of cell culture solution was added to each well. The steps were repeated three times, and the plates were incubated at 37°C in 5% CO_2. EP was diluted to 25 mmol/L, 50 mmol/L, 100 mmol/L, 150 mmol/L, 200 mmol/L, and 250 mmol/L in complete medium and then treated with drugs. Next, observation was conducted. At the end of the culture, 15 µL of MTT solution was pipetted into each well and then incubated at 37°C and 5% CO_2 in the dark for 4 h. After the incubation, the liquid in the wells was aspirated, and then, 200 µl of DMSO was added to the wells, which were shaken at room temperature for 10 min. Finally, the OD value was measured at the same time with a microplate reader at a wavelength of 492 nm, and the effect on cell proliferation was analysed based on the measured OD value. The experiment was repeated 3 times.

2.2.3. Scratch test

After the cells had grown normally into the logarithmic growth phase, the cell density was adjusted to 5×10^4 cells/ml, the cells were transferred to 6-well cell culture plates, and 1 ml of each concentration gradient was added to each well. The wells were cultured in duplicate at 37°C in a 5% CO_2 environment. When the cells were 80% confluent, a pipette tip was used to streak the cell surface, and the plate was subsequently washed with prepared PBS to remove the streaked cells. This step was repeated 3 times. Then, the cells were cultured in serum-free medium. The base was photographed. EPs were diluted with complete medium to 100 mmol/L, 150 mmol/L, and 200 mmol/L, after which the cells were treated with drugs. After culturing for 24 h, the EPs were observed under a microscope. The cells were photographed and recorded under a microscope, and the data analysis was performed using professional software. The overall distance of cell migration was calculated as the distance at 24 h divided by the distance at 0 h.

2.2.4. Grouping and dosing methods for the experimental animals

The 10 nude mice that had successfully established the model were divided into the EP group and the model group using the random number table method, with 5 mice in each group. The EP group was injected intraperitoneally with 100 mg/kg ethyl pyruvate, and the model group was injected with an equal volume of normal saline. The two groups of nude mice were dosed once a day for 14 consecutive days.

2.2.5. Observation of vital signs and determination of body weight in nude mice

During the entire dosing period, the condition of the nude mice, including their diet, drinking status and mental condition, was carefully recorded, and their body weight was measured every week. The sizes of the tumors, inclu-

ding their major diameter (a) and minor diameter (b), were measured regularly twice a week. The tumor volume and tumor inhibition rate were calculated based on the results.

2.2.6. Preparation of tumor specimens from nude mice

After 14 days of EP treatment, the nude mice were euthanized. The postoperative nude mice were photographed and recorded. After the tumor was completely removed using medical scissors and forceps, the tumor was transferred to a whiteboard and arranged in descending order from large to small for photographic records. The weight of the tumor was measured, the tumor was fixed for imaging, and the tissue was subsequently embedded in the sections.

2.2.7. Immunohistochemical analysis

Paraffin sections were dewaxed until they were hydrated. After adding 3% hydrogen peroxide, the cells were incubated at room temperature for approximately 10 min. Distilled water was used for rinsing, followed by soaking in PBS for approximately 5 min, and this step was repeated twice. After adding 10% normal goat serum (which needs to be diluted with PBS), the cells were blocked and incubated at room temperature. After 10 min, the serum was removed, and the solution was added to the serum without washing. Primary antibody working solution was added, and the samples were subsequently placed in a 4°C freezer for incubation overnight. After removal, the cells were rinsed with PBS three times for 5 min each. A certain amount of secondary antibody working solution (biotin-labelled) was added, and the mixture was incubated at 37°C for 0.5 h. After removal, the tissues were rinsed with PBS three times for 5 min each. Streptavidin working solution (labelled) was added, and the mixture was incubated at 37°C for 0.5 h. After removal, the tissues were rinsed with PBS three times for 5 min each. An appropriate amount of DAB chromogenic reagent was added for approximately 10 min. The tissues were rinsed with tap water, counterstained, dehydrated, cleared, and mounted. The samples were observed under a microscope and photographed. Three good fields were collected from each section for data analysis.

2.3. Statistical analysis

The data were statistically analysed by Statistic Package for Social Science (SPSS) 25.0 (IBM, Armonk, NY, USA) and GraphPad Prism 6.0 (La Jolla, CA, USA). The measurement data in this paper are expressed as the X ± SD and were subjected to Student's test. $P<0.05$ was considered to indicate statistical significance.

3. Results

3.1. Results of the MTT experiment

MTT assays were performed to assess the inhibitory effect of EP on the proliferation of ECAl09 cells at different concentrations. Each experiment was conducted in triplicate. The results consistently showed a decrease in ECAl09 cell proliferation as the concentration of EP increased. S-curve fitting was performed using GraphPad Prism 6.0. The IC50 of EP for the proliferation of ECAl09 esophageal cancer cells was 156.7 mM (Figure 1).

3.2. Results of the scratch test

The results obtained from the scratch experiment revealed a statistically significant difference ($P<0.05$) in the migration distance of cells among the groups after 24 hours of EP administration. Specifically, the 100 mmol/L EP, 150 mmol/L EP, and 200 mmol/L EP groups exhibited significant differences ($P<0.05$). The migration distance of cells in Group l was significantly shorter than that in the control group ($P<0.05$). Although the migration distance of cells in the 200 mmol/L EP group was significantly lower than that in the 150 mmol/L EP group, the difference was not statistically significant (Figure 2).

3.3. EP administration inhibited the growth of ESCC in nude mice

Following tumor formation in nude mice, ten mice with a tumor mass of 148 ± 5 mm3 were selected for subsequent treatment (physiologically administered saline to the model group; 100 mg/kg EP to the treatment group). Daily observations were made to monitor changes in the mice's diet, drinking water, and behavior. After drug administration, the mice in the model group exhibited slower movement, reduced diet and drinking water consumption, and an increase in tumor volume. However, the mice in the EP group exhibited normal activity levels, maintained their diet and drinking water intake, and did not experience significant weight loss. Based on the tumor volume, the EP-treated mice exhibited a 59.17% inhibition rate of tumor growth after 14 days of treatment. This inhibition

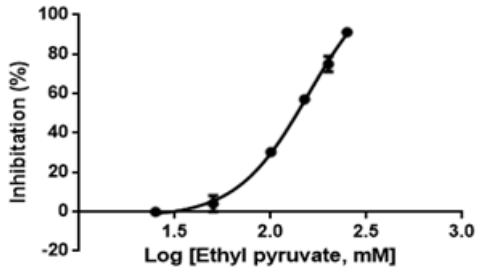

Fig. 1. Effect of EP on the proliferation of ECAl09 cells.

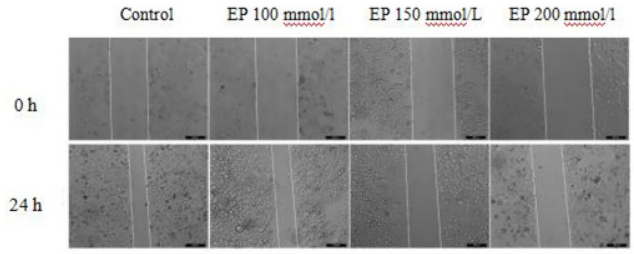

Fig. 2. Migration distance of the ECAl09 cells in each group.

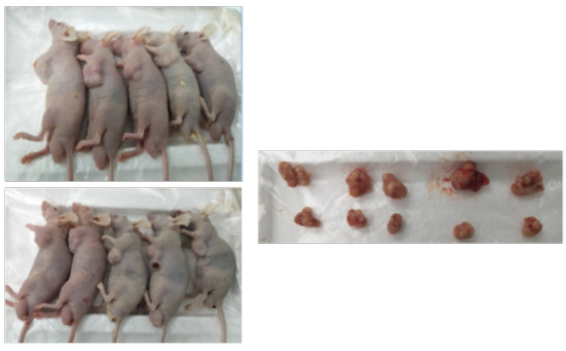

Fig. 3. Effect of EP on the tumor volume in the ESCC tumor formation model in nude mice.

rate exceeded the threshold of 40%, as stated in the Drug Efficacy Evaluation Manual, indicating effective inhibition of tumor growth by EP (Figures 3-5).

3.4. The expression of HMGB1, TLR4, and MyD88 in ESCC tissues after EP administration

Previous studies have revealed the localization of the HMGB1, TLR4, and MyD88 proteins in the nucleus, cytoplasm, cell membrane, and cytoplasm, respectively, with positive staining appearing as light yellow to brown-yellow granules [12, 13]. The results demonstrated significant differences ($P < 0.05$) in the expression of the HMGB1, TLR4, and MyD88 proteins between the EP group (30.7%, 30.1%, and 29.2%, respectively) and the model group (69.1%, 66.7%, and 69.3%, respectively) (Figure 6).

4. Discussion

ESCC is the most common histological subtype of esophageal cancer, with a global age-standardized rate (ASR) of 5.4/100,000 people in 2020. ESCC is concentrated in East Asia, South Central Asia, and South Africa [14]. Currently, surgery combined with chemotherapy and radiotherapy (CRT) is considered the standard treatment for ESCC [15]. However, the toxicity associated with chemotherapy and radiotherapy limits treatment options [15, 16]. Therefore, there is a need to explore new treatment strategies to mitigate the severity of this cancer.

Through culture experiments on ESCC cell lines, we observed that EP treatment not only suppressed cell proliferation but also significantly inhibited cell movement and migration. Additionally, our immunohistochemistry data revealed a significant reduction in the secretion of HMGB1 in the tissue culture medium after EP treatment. This change was accompanied by increased nuclear localization of HMGB1 and decreased localization of TLR4 and MyD88 in ESCC cells. We hypothesized that EP inhibits TLR4 and MyD88 activity by affecting HMGB1 release, thereby suppressing the growth of ESCC through the HMGB1/TLR4-MyD88 signaling pathway.

The HMGB1-mediated TLR4-MyD88 signaling pathway is implicated in the development of various diseases. For instance, in the acute stage of ICH, HMGB1 translocates from the nucleus to the cytoplasm, where it upregulates TLR4-MyD88 expression and induces autophagy, ultimately promoting neural dysfunction [18]. In an MRL/lpr mouse model and plasma-induced cell experiments in patients with lupus nephritis (LN), extracellular HMGB1 was found to activate the TLR4/MyD88 signaling pathway, leading to NF-κB nuclear translocation and glomerular endothelial cell damage [19]. Moreover, during HPV-mediated cell transformation, the HMGB1-TLR4/MyD88-SARM1 signaling axis is involved in evasion of the innate immune system, thereby increasing the likelihood of cervical cancer due to persistent HPV infection [20]. Our previous studies also confirmed that TLR4 overexpression in ESCC cells can mitigate the inhibitory effects of HMGB1 or TLR4 knockout on TLR4-MyD88 signaling and epithelial-mesenchymal transition (EMT) markers and reverse the effects of HMGB1 or TLR4 knockout on the proliferation, migration, and invasion of ESCC cells. As an HMGB1 antagonist, EP can reduce the severity of related diseases by inhibiting the HMGB1/TLR4-MyD88 signaling pathway. For example, EP has been reported to reduce the expression of proinflammatory cytokines such as IL-1β, IL-6, and TNF-α by inhibiting the HMGB1/TLR4/MyD88/pNF-κB p65 signaling pathway, thereby attenuating LPS-induced intestinal inflammation [21]. EP has also been demonstrated to have a protective effect on SCI by inhibiting the HMGB1/TLR4/NF-κB signaling pathway [22]. In our study, we observed for the first time that EP may suppress the development of ESCC by downregulating the HMGB1/TLR4-MyD88 signaling pathway. Although the specific mechanism involved remains unclear, our results showed that EP exhibited significant antitumour activity in both in vitro and in vivo ESCC models. This difference may be attributed to the disruption of the HMGB1-TLR4 autocrine loop.

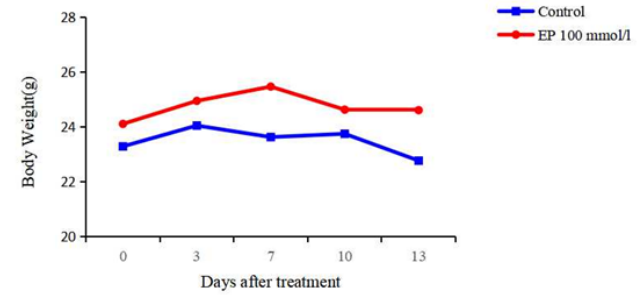

Fig. 4. Effect of EP on the body weight of nude mice in the ESCC tumor formation model.

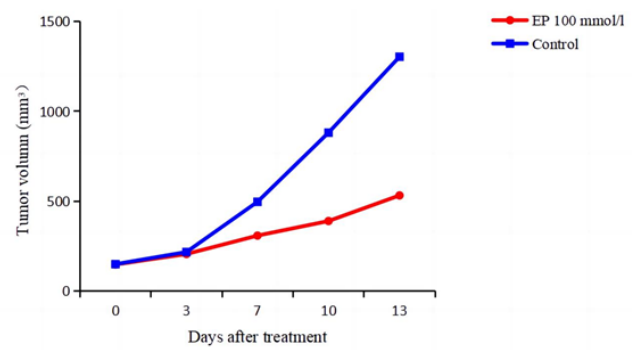

Fig. 5. Effect of EP on tumor volume in the ESCC tumor formation model in nude mice.

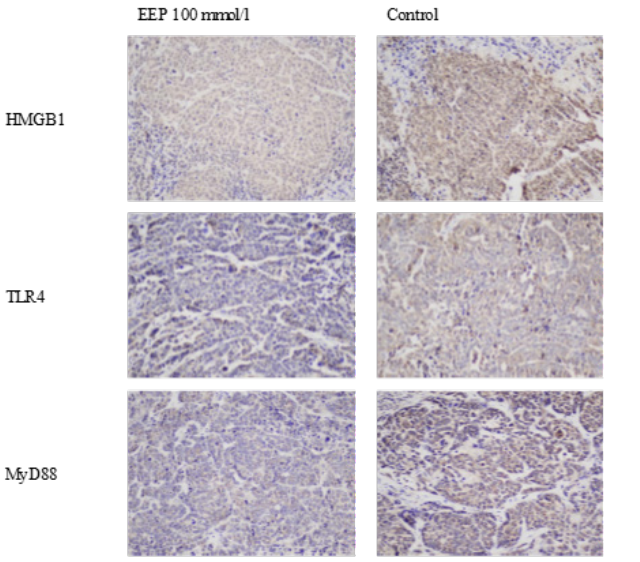

Fig. 6. Effect of EP on the protein expression of HMGB1, TLR4, and MyD88 in the ESCC tissues of nude mice.

Unlike other compounds that inhibit or affect HMGB1, EP is not associated with host toxicity [23], as confirmed in animal model experiments [24]. Furthermore, EP has passed safety tests in clinical human trials [23]. Recent clinical experiments have shown that EP can enhance tumor cell sensitivity to various antitumour drugs. For instance, EP in combination with treatment reduced multidrug resistance and enhanced the sensitivity of hepatocellular carcinoma (HCC) to sorafenib [25]. Additionally, the combination of recombinant human milk peptide neptine lactate (RL2) and ethyl pyruvate produced a better long-term antitumour response in combination with MX-7 rhabdomyosarcoma cells than did RL2 alone [26]. EP, an HMGB1 inhibitor, has also been shown to enhance the sensitivity of liver cancer cells (HepG2 cells) to CP through the inhibition of HMGB1 [27]. Moreover, EP can provide certain protection to normal cells and mitigate excessive radiotoxicity and cytotoxicity caused by radiotherapy and chemotherapy [28-30]. Although our in vivo experiments did not reveal a significant difference in the survival rate of the mice, the results confirmed that EP administration effectively inhibited tumor growth and had minimal toxic side effects on the mice during treatment, thereby effectively prolonging their survival. In summary, our study supports the potential of EP as an adjuvant therapy for ESCC.

This study has several limitations. First, HMGB1 mediates the proliferation and metastasis of ESCC through multiple signaling pathways, and interplay may occur between different signaling pathways. In this study, we observed the effect of EP inhibition of only the HMGB1/TLR4-MyD88 signaling pathway on ESCC growth. However, further research is needed to explore the regulatory effect of EP on HMGB1-mediated signaling pathways leading to ESCC. Second, in addition to HMGB1, other factors, such as the hypoxic environment, virulence factor (LPS), and danger-associated pattern molecule (DAMP), such as S100A8/A9, may promote ESCC cell proliferation through the TLR4 pathway. This study did not investigate whether EP inhibits ESCC development through other forms of the TLR4 pathway directly or indirectly. Third, previous studies have shown a synergistic effect between EP and other HMGB1 inhibitors in antitumour treatment. For example, triptolide significantly reduced the expression levels of cytoplasmic HMGB1 and TLR4 and, in combination with EP, inhibited the growth of breast cancer cells [31]. This study did not explore direct efficacy comparisons between EP and other HMGB1 inhibitors in treating ESCC.

5. Conclusions

In recent years, EP has demonstrated superior efficacy in animal models of malignant tumors by inhibiting tumor growth and improving overall survival rates. Our study is the first to investigate the association between EP and ESCC, and the results reveal its potent antitumour activity in both in vitro and in vivo ESCC models. These findings could lead to the use of additional treatment options for ESCC.

Conflict of interests

The author has no conflicts with any step of the article preparation.

Consent for publications

The author read and approved the final manuscript for publication.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Lianyungang Hospital affiliated with Xuzhou Medical University.

Informed consent

The authors declare that no patients were used in this study.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions

Xuyang Liang and Lu Wang designed the study and performed the experiments, Jing Wen and Jing Xu collected the data, Zhimei Zhang and Shuxian Zhang analyzed the data, Xuyang Liang and Lu Wang prepared the manuscript. All authors read and approved the final manuscript.

Funding

This work was supported by the Lianyungang City Science and Technology project (SH1532) “The role of HMGB1/TLR-MyD88 signaling pathway in the pathogenesis of esophageal cancer”.

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