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Original Article

Suppression of long-circulating and liposomes containing gefitinibon nasopharyngeal carcinoma cells

Jianxin Di[1], Hongyan Zhang[2], Hongxia Hui[3]

Departments of [1]Otorhinolaryngology, [2]Department of Oncological Radiotherapy, General Hospital of Tianjin Medical University, Tianjin 300052, P. R. China; [3]Department of Medical Oncology, Huai'an First People's Hospital, Nanjing Medical University, Huai'an 223300, Jiangsu, P. R. China

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Abstract: Objective: To investigate the suppression of long-circulating and liposomes containing gefitinib (LCLCG) against nasopharyngeal carcinoma cells. Methods: LCLCG was prepared, and its loading capacity and encapsulation rate were analyzed and determined by the low-speed centrifugation. The stability of the modified liposome formulation was verified by comparing the changes of particle size and zeta potential of liposomes in dynamic blood in an invitro simulated blood circulation system (in serum albumin phosphate solution at 37°C). HONE1 (a human nasopharyngeal carcinoma cell line) was treated with LCLCG at different concentrations and the cell viability was assessed by the MTT assay at different time periods after treatment. The influence of LCLCG on apoptosis of HONE1 was analyzed by using the Hoechst 33258 fluorescence staining while the effect of LCLCG on metastasis of HONE1 was detected by the scratch-wound assay. Results: The encapsulation of Gefitinib with liposome was determined to be a success, characterized by high stability, with drug loading rates of (7.14 ± 0.7)% and the encapsulation rates of (86.7 ± 12.1)%. The MTT assay results showed that LCLCG could suppress the proliferation of HONE1 in a dose-and-time-dependent manner. The Hoechst 33258 fluorescence staining results indicated that LCLCG could induce apoptosis of HONE1. The scratch-wound assay results revealed that LCLCG could suppress the metastasis of HONE1. Conclusion: Preliminary results in this study showed that LCLCG was effective in inducing apoptosis of HONE1 and suppressing against their proliferation and migration; therefore, it is expected to become a novel potent therapy for nasopharyngeal carcinoma.

Keywords: Gefitinib, long-circulating and liposomes containing gefitinib, nasopharyngeal carcinoma, suppression

Introduction

Nasopharyngeal carcinoma (NPC) is head and neck malignant carcinoma with a high incidence in the southeast area of China. Up till now, 98% of the nasopharyngeal carcinoma cases belong to poorly differentiated keratinizing squamous cell carcinoma (SCC), a subtype of NPC. Studies have shown the etiology of NPC has been closely related to epstein-barr virus (EBv) infection, genetic susceptibility and environmental factors [1]. At the early stage, NPC patients generally present no specific clinical symptoms. Instead, the majority of them have already been at the moderate or advanced stages when they are diagnosed definitely. Clinically, radiotherapy complementary with chemotherapy is a major treatment modality for the patients [3, 4]. However, this modality is not targeting. While controlling and killing the tumor cells it may cause serious destruction and damage to normal tissues, induce various complications, and even result in the onset of secondary tumors [5]. In addition, with the development and progress of medical detecting techniques, an increasing number of patients with nasopharyngealcarcinoma have been diagnosed at the early stage [6]. Therefore, the current clinical and basic medical research should focus on how to further improve the antitumoractivity against nasopharyngeal carcinoma and minimize the toxicity.

Liposome is a novel carrier for targeted agents, whose lipid bilayer molecular structure is similar to that of a cell, which can be delivered to the targeted lesion sites through the vascular endothelial cells [7]. In addition, the ability of

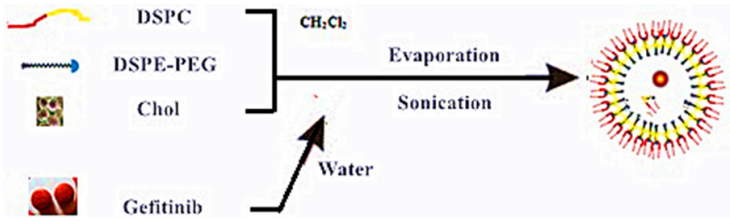

Figure 1. Flow chart for LCLCG preparation.

liposomes to change the distribution of encapsulated agents in the body induces more of them to aggregate and release in the primary lesion sites, so fewer agents would release in other normal tissues, which can improve the efficacy of agents agents and minimize the toxicity on normal tissues, realizing the goal of targeted agent delivery [8-10]. The long-circulating and liposomes containing gefitinib (LCLCG), a novel liposome formulation developed in recent years, compared with conventional liposomes, can avoid being phagocytized by macrophages in the reticuloendothelial system and prolong the circulation time for the agents acting on the tissues, so as to improve the efficacy of the agents [11].

In this study, we encapsulated gefitinib in long circulating liposome to prepare LCLCG administered it to HONE1 (Nasopharyngeal carcinoma cell line) to determine the influence of LCLCG on development, metastasis and apoptosis of HONE1. The findings of this study will bring new insight into the development of agents and planning of treatment modalities for nasopharyngeal carcinoma.

Materials and methods

Materials and reagents

Distearoyl Phosphatidylcholine (DSPC) and distearoyl phosphatidyl ethanolamine-Polyethylene glycol (DSPE-PEG2000) were purchased from Avanti Polar Lipids in the USA, Cholesterol (Chol) and other conventional reagentsfrom Chemical Reagent Co., ltd in China and HONE1 (nasopharyngeal carcinoma cell line) from the ATCC Cell Library.

Preparation ofLCLCG

DSPC (6.7 mg), DSPE-PEG2000 (1.7 mg) and Chol (6.5 mg) were weighed and mixed into a round bottom flask (100 mL) and then Gefitinib/PBS solution (6 mL) was prepared for use by mixing gefitinib solution (0.1 mL, at the serum concentration of 50 mg/mL) with PBS solution. The Ultra-sonication was performed following 3 mL of dichloromethane (CH_2Cl_2) was poured into the flask. Meanwhile, gefitinib/PBS solution (6 mL) was continuously added into it. After the ultra-sonication process, LCLCG was produced by removing CH_2Cl_2 with a rotary evaporator and then stored for use after being fully washed in PBS solution for 3 times. LCLCG was prepared in the procedure as shown in **Figure 1**.

Calculation of encapsulation rate and agent loading capacity

First, after 2 ml of Methyl alcohol (heated at 60°C, which was good for demulsification) was added into the 0.5 mL of centrifuged liposome suspensions (at 3000 g *10 min), the suspensions were demulsificated until the liposome solution became completely clear, which was used for determining the encapsulated agent content. Next, the same amount of non-centrifuged liposome suspensions (0.5 mL) were demulsificated in the same way and used for determining the total agent concent. Finally, the total encapsulated agent concentration was determined in accordance with the Standard Curve. The formulas are shown as follows: encapsulation rate = encapsulated drug content/total drug content × 100%; and agent loading capacity = encapsulated drug content/weight of nano-liposome × 100%.

LCLCG stability test

Liposome is generally administered intravenously. As a variety of factors in the blood like phospholipase, high-density lipoprotein and the complement system undermine the liposomes, a hydrophilic pathway forms in the phospholipid membrane, leading to leakage of coated agents and infiltration of water and electrolytes. That in turn further accelerates liposome clearance [3, 4]. In this study, the interactions of liposomes, serum and plasma were investigated by detecting changes of particle size and zeta potential of liposomes in dynamic blood in an in vitro simulated blood circulation system (in serum albumin phos-

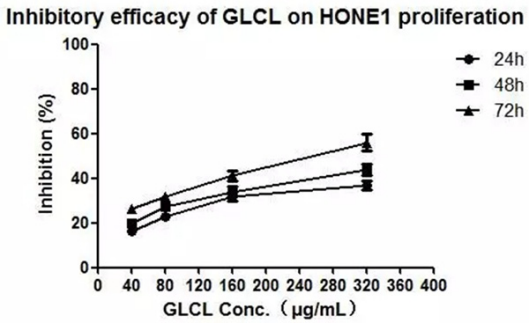

Figure 2. Suppressive effects of LCLCG on proliferation of HONE1. The suppression of LCLCG on proliferation of HONE1 was expressed in a concentration-and-time-dependent manner. When LCLCG was administered at 320 g/mL at 72 h, its inhibitory rates range 56.05 ± 3.66%.

phate solution at 37°C) at 12 h, 24 h and 48 h respectively to evaluate the stability of LCLCG.

Treatment of HONE1 (a nasopharyngeal carcinoma cell line)

The mononuclear cell suspensions at $1.5*10^4$/mL were prepared by plating HONE1 (nasopharyngeal carcinoma cell line) in the exponential growth phase into RPMI-1640 culture medium and seeded into a 96-well culture plate at 200 μL/well (about 3000 cells per well) and then incubated in an incubator at 37°C in an atmosphere with 5% CO_2 for 24 h. After that, the culture medium was removed from the suspensions. The suspensions were co-cultured by adding LCLCG culture medium at distinctive concentrations (40, 80, 160, 320 μg/mL) respectively, six repeated wells at each concentration. Meanwhile, the negative control groups (culture medium without LCLCG) and the experimental groups (unencapsulated gefitinib culture medium) were set for comparison.

Cell proliferation determined by the MTT assay

The influence of LCLCG on proliferation of HONE1 was detected by the MTT assay. After LCLCG was treated at 24 h, 48 h and 72 h respectively, 20 μL of MTT reagents at 5 mg/mL were added into each experimental well followed by another 4 h culture in the incubator. Then the supernatant was extracted carefully from the medium. After that, DMSO 150 μL was added into each experimental well. HONE1 cells were oscillated at low speed without exposure to light at room temperature for 10 min till fully dissolution of the purple crystals. OD490 value was measured by the ELIASA (ELX808, USA) and the suppressive rates of LCLCG against HONE1 at distinctive concentrations were calculated in accordance with the following formula (All the experiments were repeated in triplicates):

Suppressive rate (%) = (1-OD mean value of the treated group/OD mean value of the negative control group) × 100%.

Apoptosis of cells determined by the hoechst 33258 fluorescence staining assay

The sterile coverslips were plated in a 6-well culture plate to inject the HONE1 in the exponential growth phase for the purpose of preparing cell glass coverslips which were then inoculated in the incubator at 37°C in an atmosphere with 5% CO_2 till 70-80% of the coverslips were covered with adherent cells and then the cells were treated by LCLCG at distinctive concentrations and the negative control groups were set up. By contrast, the treated groups selected LCLCG at 40, 80, 160 and 320 g/mL, respectively. After 48 h culture, the cells were extracted away the culture medium, washed in PBS solution for 3 times and then fixed by adding 0.5 mL of Polyoxymethylene (stationary liquid) at 4% for 30 min. After Polyoxymethylene (stationary liquid) was cleaned away, the cells were washed in the PBS solution for 3 times and then assessed by adding some Hoechst 33258 staining solution for 10 min, and again washed in PBS solution for 3 times. After natural drying, the coverslips were sealed by neutral Glycerol and then observed under a fluorescence microscope (Olympus CKX41, Japan) at 10 times magnification and photographed.

Cell scratch-wound assay

HONE1 cells in the exponential growth phase were seeded in a 6-well culture plate and then inoculated in an incubator at 37°C in an atmosphere with 5% CO_2. After the cells proliferated to 80-90% confluence, the pipette (1 mL) sterilized in an autoclave was used to draw out even scratches on the cells while its tip maintained perpendicular to the bottom of the culture plate. The cells were washed in PBS solution for 3 times, into which serum-free medium was added. Under an inverted microscope, the cells were marked, photographed at idea mag-

Table 1. Comparison of suppression on HONE1 between LCLCG and unencapsulated gefitinib

Time (h)	Concentration (ug/ml)	LCLCG	Unencapsulated gefitinib	t	P
24 h	40	18.7	3.52	2.31	<0.05
	80	19.9	5.71	4.72	
	160	30.2	9.21	5.03	
	320	32.1	15.3	3.11	
48 h	40	20.1	4.78	2.81	<0.05
	80	29.8	9.26	5.01	
	160	32.4	13.81	5.62	
	320	40.3	16.31	3.22	
72 h	40	26.1	7.52	5.31	<0.05
	80	32.3	13.58	6.02	
	160	40.5	18.97	5.98	
	320	49.9	22.39	3.67	

Note: P<0.05 is considered statistically significant.

nification and the initial graphics were recorded. Then the cells were treated with LCLCG at different concentrations and the native control group was set up. The treat group chose LCLCG at 40, 80, 160 and 320 μg/mL, respectively. After 48h of cell culture, under an inverted microscope (Olympus CKX41, Japan) the cells were observed at the marked magnification and photographed. All experiments were repeated in triplicates.

Statistical analysis

SPSS 18.0 software program was used for statistical analysis. The measurement data were expressed as mean ± standard deviation. The comparison among groups was analyzed by the One-way ANOVA assay while the comparison between groups was detected by the LSD-t assay. P<0.05 was considered statistically significant.

Results

Stability, agent loading and encapsulation rate of LCLCG

The experimental results have shown that there were no significant changes of particle size and zeta potential of liposomes in dynamic blood in an in vitro simulated blood circulation system (in serum albumin phosphate solution at 37°C). HONE1 (a human nasopharyngeal carcinoma cell line) and LCLCG has high stability in its physical, chemical, biological properties. The calculated loading rates of LCLCG ranged (7.14 ± 0.7)% and encapsulation rates were (86.7 ± 12.1)%, indicating excellent agent loading and high encapsulation rate of LCLCG.

Suppression of LCLCG on proliferation of HONE1

The MTT results showed that the suppression of LCLCG on proliferation of HONE1 gradually increased with the rise in LCLCG concentration. In particular, with LCLCG at the same concentration, the suppression of LCLCG on proliferation of HONE1 gradually increased with the extension of circulation time, suggesting that suppression of LCLCG on proliferation of HONE1 was expressed in a concentration-and-time-dependent manner. When LCLCG was administered at 320 μg/mL, its circulation time was up to 72 hours and its suppressive rates ranged (56.05 ± 3.66)%. Currently, impressive rate less than 30% is considered as low sensitivity, 30%-50% as moderate, and higher than 50% as high [12]. The results of this study suggested that HONE1 was high sensitive to LCLCG at 320 μg/mL at 72 h (**Figure 2**). Under the same conditions, in the unencapsulated gefitinib control group, the unencapsulated gefitinib reached the targeted tissues of nasopharyngeal carcinoma cells at 320 μg/mL at 43 h, with suppressive rates ranged (20.11 ± 4.66)%. The results of statistical analyses were statistically significant (P<0.05) (**Table 1**).

Influence of LCLCG on apoptosis of HONE1

Nuclear Chromatin condensation is morphologically typical change of cell apoptosis. Under the conditions of the same staining period, exposure period and dye concentration, the Hoechst 33258 fluorescence staining analysis showed that the nuclei in the negative control group were normally blue, showing intact membrane, round or oval shape and even distribution. In contrast, with the increase of LCLCG concentration, the nuclei of HONE1 began to condense, showing smaller size, uneven distribution, dense and thick staining and being bright blue (**Figure 3**). Further analyses showed that with the increase of LCLCG concentration, the number of apoptotic cells gradually grew. When the concentration of LCLCG ranged

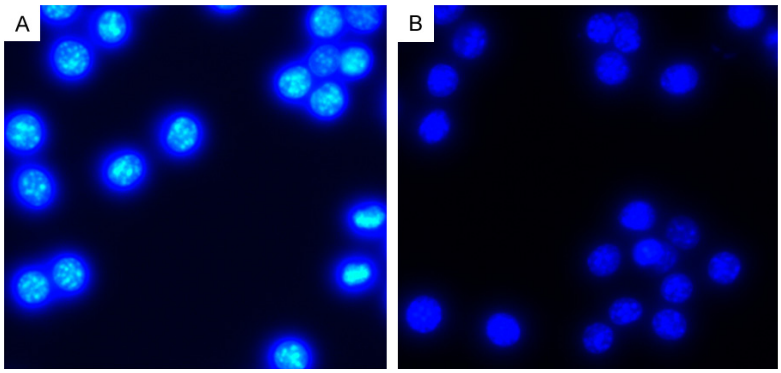

Figure 3. Apoptosis of HONE1 analyzed by the Hoechst 33258 fluorescence staining assay. A. After HONE1 was treated by LCLCG at 320 μg/mL for 48 h, the Hoechst 33258 fluorescence staining results shows the nuclei becomes condensed, showing smaller size, uneven distribution, and bright blue color. B. The nuclei from the negative control group are normally blue, showing intact membrane, round or oval shape and even distribution (× 400).

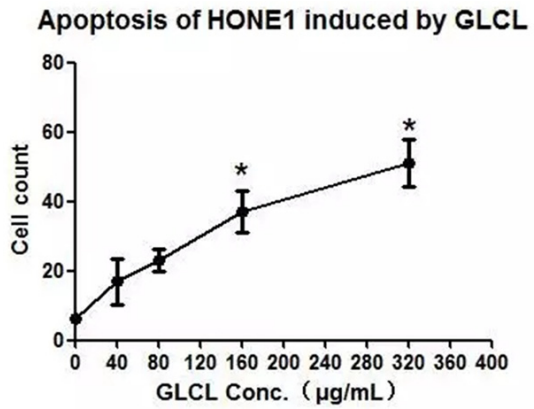

Figure 4. Influence of LCLCG on apoptosis of HONE1 at distinctive concentrations. With the increase in concentration of LCLCG, the number of apoptotic cells increases gradually. When the concentrations of LCLCG range between 160 μg/mL and 320 μg/mL, the number of apoptotic cells is significantly larger than that of the negative control group, so the difference is statistically significant ($P<0.05$). *: compared with the negative control group (LCLCG 0 μg/mL) ($P<0.05$).

from 160 μg/mL to 320 μg/mL, the number of apoptotic cells became significantly larger than that of the negative control group, and the difference was statistically significant ($P<0.05$) (**Figure 4**).

Influence of LCLCG on migration of HONE1

As shown in **Figure 5**, the scratch-wound assay results reveal that LCLCG exerts suppressive effect on migration of HONE1. With the increase in the concentration of LCLCG, its suppressive effect is more obvious. In particular, when the LCLCG concentration is equal to or larger than 160 μg/mL, the scratch-wound healing was significantly slower.

Discussion

Nasopharyngeal carcinoma is one of the malignant tumors with high incidence in China, ranking first among head and neck malignant tumors. The disease tends to be more prevalent in Guangdong, Guangxi, Fujian and other places. In recent years, its incidence has been increasingly higher. The occurrence and development of the disease are influenced by various factors, so how to cure the disease has been a trying problem in the medical world. Compared with the conventional medication, the targeted therapy is better-targeted, more effective, safer and less toxic. It has become the leading trend in tumor treatment.

EGFR molecular-targeted therapy is one of the most prominent studies on tumor treatment [13]. Studies have shown that the expression level of epidermal growth factor (EGF) in non-keratinizing nasopharyngeal carcinoma is high up to 90% [14], and there was amplification of epidermal growth factor receptor (EGFR) gene in tumor tissues of patients with nasopharyngeal carcinoma [15]. In the EGFR targeted therapy, gefitinib is a more mature targeted agent. The results have showed that gefitinib can play a role in suppressing the proliferation of a variety of solid tumors, inducing apoptosis of tumor cells, preventing tumor metastasis and causing arrest of specific cell cycle [16]. Compared to previous studies, in our study, we took advantage of the agent delivery system of LCLCG carrier through which the agents can be encapsulated in the carrier to escape being scavenged by phagocytes in the reticuloendothelial system through polyethylene glycol (PEG) on the surface of the carrier, realizing efficacy of tumor targeted treatment enhanced by the infiltration of the solid tumor angiogenesis. LCLCG is characterized by low toxicity, high efficacy and multi-targeted synergistic effects

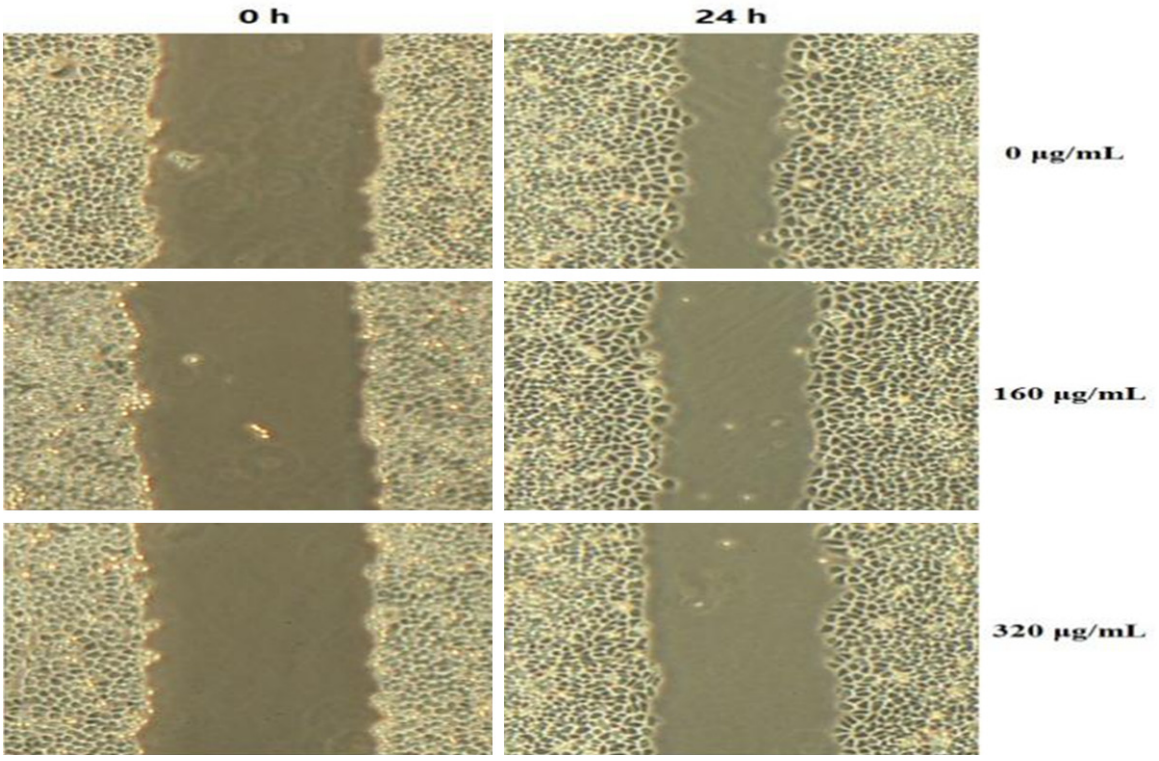

Figure 5. Influence of LCLCG on migration of HONE1. LCLCG exerts suppression on metastasis of HONE1. In particular, when the LCLCG concentrations are equal to or larger than 160 μg/mL, the scratch-wound healing is significantly slower.

[17], taking advantage in improving the tumor microenvironment and enhancing antitumor efficacy [18].

In previous studies, liposome was generally administered intravenously. However, as a variety of factors in the blood like phospholipase, high-density lipoprotein and the complement system undermine the liposomes, a hydrophilic pathway forms in the phospholipid membrane, leading to leakage of encapsulated agents and infiltration of water and electrolytes which further accelerates liposome-clearance [19, 20]. In this study, the interactions of liposomes, serum and plasma were investigated by detecting changes of particle size and zeta potential of liposomes in dynamic blood in an in vitro simulated blood circulation system (in serum albumin phosphate solution at 37°C) at 12 h, 24 h and 48 h respectively to evaluate the stability of LCLCG. The results shows that there were no significant changes in physical, chemical and biological properties of LCLCG in thedynamic blood in an in vitro simulated blood circulation system ($P>0.05$), suggesting that LCLCG has high stability. According to the preliminary analytic results of drug loading and encapsulation of LCLCG by the low-speed centrifugation, the drug loading of LCLCG ranged (7.14 ± 0.7)% and the encapsulation rates (86.7 ± 12.1)%, suggesting LCLCG has high drug loading and encapsulation rates. It proved that our LCLCG preparation is successful. In the process of exploring the impacts of CLCL on the biological behavior of HNE1, the in vitro studies showed that LCLCG in vitro could suppress growth, proliferation and migration of HONE1 in a dose-time-dependent manner. When LCLCG was administered at 320 μg/mL at 72 h, its suppressive rates ranged (56.05 ± 3.66)%. The unencapsulated gefitinib in the controls showed the defects of high concentration of effective agents, short circulation and low suppressive rates while LCLCG had a higher targeted effect on apoptosis of HONE1, characterized by low blood concentration, prolonged circulation time and high suppressive rate, further proving LCLCG had significant antitumor activity.

In this study, however, there are still some limitations: firstly, we investigated the influence of

LCLCG on biological behavior of nasopharyngeal carcinoma cells only through in vitro trials and made a preliminary study on concentration and time for LCLCG activity; but the data failed to be validated by the tumor-bearing mice model, the optimal dose and time for LCLCG activity remains to be investigated further invivo experiments and clinical trials. Secondly, while LCLCG has antitumor-targeted effect, its adverse effects on the normal tissues need confirming in vivo experiments in the future. Finally, further investigations of antitumor-specific molecular mechanisms of LCLCG are in the air.

In conclusion, the preliminary results of this study illustrate LCLCG can induce apoptosis of HONE1 and suppress their growth and metastasis. Therefore, LCLCG is a promising new therapeutic modality for nasopharyngeal carcinoma, while the mechanisms for molecular targeted therapy of LCLCG are necessary to be further investigated.

Disclosure of conflict of interest

None.

Address correspondence to: Hongxia Hui, Department of Medical Oncology, Huai'an First People's Hospital, Nanjing Medical University, No. 6 Beijing West Road, Huai'an 223300, Jiangsu, P. R. China. Tel: +86-517-80872630; Fax: +86-517-80872630; E-mail: drhuihongxia@163.com

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