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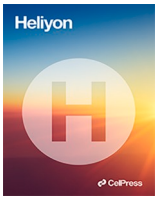

Research article

Regulation of Dihydroartemisinin on the pathological progression of laryngeal carcinoma through the periostin/YAP/IL-6 pathway

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ABSTRACT

Objective: Laryngeal cancer (LC) is one of the most common squamous cell carcinomas of the head and neck in clinical practice, and its incidence has been increasing in recent years, but the prognosis of the patients is not favorable. Hence, it is critical to re-understand and deeply study the causes and mechanisms of LC and explore new effective treatment methods and strategies. In this study, we analyzed the effect of Dihydroartemisinin (DHA) on the pathological progression of LC through the periostin (POSTN)/Yes-associated protein (YAP)/interleukin (IL)-6 pathway, which can provide new clinical references and guidelines.

Methods: POSTN, YAP, and IL-6 levels in 18 pairs of fresh LC tissues and adjacent counterparts in our hospital were detected. Additionally, LC TU686 cell line was purchased for DHA treatment of various concentrations to detect changes in cell biological behavior. Finally, we built a tumor-bearing mouse model with C57BL/6 mice and intragastrically administrated DHA to the animals to observe the growth of living tumors and to measure POSTN, YAP, and IL-6 expression in tumor tissues.

Results: As indicated by PCR, Western blotting, and immunohistochemistry, POSTN, YAP, and IL-6 presented higher expression in LC tissues than in adjacent counterparts. In cell experiments, the cloning rate of LC cells decreased and the apoptosis rate increased after DHA intervention, with 160 μmol/L DHA contributing to the most significant effect on LC activity inhibition. Furthermore, DHA-intervened cells exhibited markedly reduced POSTN, YAP, and IL-6 levels. Finally, the tumorigenesis experiment in nude mice showed inhibited tumor growth after DHA administration. And consistently, the expressions of POSTN, YAP, and IL-6 in living tumors decreased.

Conclusions: DHA can inhibit POSTN/YAP/IL-6 transduction, accelerate LC cell apoptosis, and alleviate the malignant progression of LC.

1. Introduction

Laryngeal carcinoma (LC) , one of the most common head and neck squamous cell cancers clinically, has a global incidence of about 2.1/100,000 that accounts for 1–5% of all tumors and a rising incidence in recent years, in clinical practice, it is believed that genetic

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factors, poor lifestyle habits, frequent consumption of spicy foods and many other reasons may lead to the occurrence of LC [1,2]. LC is mainly categorized into supravocal cancer, infravocal cancer, and vocal cord cancer, and its pathogenesis has not yet been fully clarified. The disease usually presents no obvious special clinical symptoms rather than throat discomfort and hoarseness at the initial stage, resulting in the miss of the best treatment period in most patients [3,4]. The appearance of obvious dysphagia and dyspnea usually indicate disease progression to the advanced stage, at which time the mortality of patients has greatly increased, with over 50% of them dying in 5–10 years [5]. Despite continuous progress in clinical surgery and comprehensive treatment for LC in recent years, the prognostic survival of LC patients has not been significantly improved [6]. Hence, it is critical to re-understand and deeply study the causes and mechanisms of LC and explore new effective treatment methods and strategies.

Dihydroartemisinin (DHA) is the first generation derivative of artemisinin, which has been widely used in clinical antimalarial therapy due to its excellent efficacy and low toxicity [7]. DHA has been recently indicated to exert excellent anticancer effects in malignant neoplastic diseases such as liver and ovarian cancers [8,9], but its role in LC needs further exploration. In a recent study by Sun Y et al., DHA is reported to block interleukin (IL)-6-triggered epithelial-mesenchymal transition (EMT) and laryngeal squamous cell carcinoma (LSCC) invasion, during which DHA increased miR-130b-3p expression to reduce the activation of the IL-6/STAT3 and β-catenin axis [10]. All these findings provide new insights into strategies to inhibit or even prevent LSCC metastases, but more research is needed to understand exactly how DHA affects LC.

Reviewing research on the mechanism of DHA, we found that Yes-associated protein (YAP) is an extremely important key protein. Existing evidence has confirmed that DHA promotes the anti-PD-1 effect of hepatocellular carcinoma through YAP [11]. As an important fibroblast-related cytokine in malignant tumors, YAP, together with periostin (POSTN) and IL-6, which are its upstream and downstream proteins, respectively, constitute an important signal transduction pathway that has been confirmed to be involved in the onset and development of many malignancies such as colorectal and gastric cancers [12–14]. Therefore, DHA is also speculated to affect the biological behavior changes of LC through this signaling pathway.

To testify the conjecture, this study carried out in vitro analysis of LC cells to further confirm the pathway of action of DHA in LC, so as to provide a more comprehensive and reliable reference and guidance for the future clinical application of DHA and lay a foundation for subsequent studies.

2. Materials and methods

2.1. Sample source

Eighteen pairs of fresh LC and adjacent tissues were collected from LC patients admitted to our hospital between April 2020 and October 2022. Inclusion criteria: The patients included had not received any preoperative chemoradiotherapy or antibiotic therapy and were confirmed as LC by postoperative pathological examination, with complete removal of the tumor, complete follow-up data, and no distant organ metastasis as confirmed by preoperative imaging examination. Exclusion criteria: other malignancies, post-operative LC recurrence, or death within 2 months after surgery. The Ethics Committee of our hospital has approved this study and all patients who provided the samples signed informed consent.

2.2. PCR

Total RNA was extracted following the TRIzol kit (Thermo Fisher Scientific) instructions, and POSTN, YAP, and IL-6 mRNA levels were quantified by qRT-PCR after reverse transcription of the total RNA into cDNA. Reaction conditions: The samples were pre-denaturated at 95 °C for 30 s, denaturated at 95 °C for 10 s, annealed at 60 °C for 30 s, and extended at 60 °C for 30 s. We used GAPDH as an internal reference and calculated gene expression with $2^{-\triangle\triangle CT}$. The primer sequences were designed and constructed by Shanghai Diying Biotechnology Co Ltd (Table 1).

2.3. Western blotting (WB)

The sample supernatant was collected using a RIPA lysis solution (Thermo Fisher Scientific), and the protein was electroblotted to a polyvinylidene fluoride (Shanghai Yongke Biotechnology Co.) membrane (0.45 μm) by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Sigma Aldrich). After cutting the membrane, the bands were sealed in 5 % skim milk for 2 h, and then POSTN (Abcam, ab231130), YAP (Abcam, ab205270), IL-6 (Abcam, ab233706), and GAPDH (Abcam, ab8245) primary antibodies (1:3000) were added for a night-long incubation at 4 °C. The next day, the bands were immersed in a HRP secondary antibody (1:5,000, Abcam, ab288151) to incubate at 37 °C for 1.5 h, followed by development with a chemiluminescent imager. Finally, we determined the gray

Table 1
Sequence of primers.

	F (3′-5′)	R (3′-5′)
POSTN	GCTATTCTGACGCCTCAAAACT	AGCCTCATTACTCGGTGCAAA
YAP	TAGCCCTGCGTAGCCAGTTA	TCATGCTTAGTCCACTGTCTGT
IL-6	ACAACCACGGCCTTCCCTACTT	CACGATTTCCCAGAGAACATGTG
GAPDH	GCAAAGTGGAGATTGTTGCCAT	CCTTGACTGTGCCGTTGAATTT

values of each group of protein bands with an Image J image analysis software.

2.4. Immunohistochemistry (IHC)

The dewaxed specimens were hydrated with gradient alcohol, after which antigen retrieval was performed with citric acid repair solution. Fetal bovine serum (3 %) was then added dropwise for incubation, followed by the addition of POSTN (Abcam, ab223194), YAP (Abcam, ab225440), and IL-6 (Abcam, ab9324) of corresponding concentrations for incubation at 4 °C overnight. The next day, the biotin-labeled secondary antibody was dripped for 30 min of room temperature culture. Then came staining by the strept avidin-biotin complex (SABC) method and developed by DAB (Beijing Jinjie Biotechnology Co.). After 2 min of hematoxylin restaining, the samples were dehydrated, transparentized, and mounted for the final counting and recording of the percentage of stained cells microscopically.

2.5. Cell samples

Human LCTU686 cells, ordered from Beijing BeNa Culture Collection, were immersed in a complete medium composed of 90% RPMI-1640 + 10% FBS, subcultured according to the supporting instructions, and stored in a constant temperature (37 °C) incubator with 5% CO_2.

2.6. DHA solution preparation

DHA was supplied by Tokyo Chemical Industry (Japan). 1g of DHA was precisely weighed, and DMSO was added to prepare DHA solutions with concentrations of 20, 40, 80, and 160 μmol/L, respectively.

2.7. Cell intervention

TU686 cells grown to the logarithmic (Log) phase were randomized into control group as well as groups A, B, C, and D, in which groups A-D were intervened by 20, 40, 80, and 160 μmol/L of DHA, respectively. The original culture medium was discarded and DHA solution was added, with the medium changed after 24 h. The control cells were treated with the same amount of DMSO. Cellular POSTN, YAP and IL-6 levels were measured by PCR and WB.

2.8. Flow cytometry (FCM)

Cell suspensions were placed into 6-well culture plates for 24 h, and the cells were gathered after supernatant removal and three rinses with precooled phosphate buffer saline (PBS). Following resuspension with 100 μL binding buffer, the cells were placed into AnnexinV and prodium iodide (PI) (Shanghai Beibo Biotechnology Co.) in turn for 15 min of light-tight incubation and the subsequent apoptosis detection by FCM.

2.9. Cell cloning

Cells in each group were inoculated into 6-well culture plates (500 cells/well) for continuous culture, with the medium changed every other day. The culture was terminated when there were macroscopic cell colonies in the plates. After 4 % paraformaldehyde fixation for half an hour, the cells were treated with a 30-min staining with 1 % crystal violet, drying, photographing, and the final cell colony counting.

2.10. Cellular immunofluorescence

Following routine pancreatin digestion, the Log-growth-phase cells were counted and prepared as 4×10^5 cells/mL using a complete culture medium for inoculation in six-well culture plates. After the cells grew on glass coverslips, PBS was used to wash them thrice, followed by overnight incubation with POSTN (Abcam, ab79946), YAP (Abcam, ab114862), IL-6 (Abcam, ab290735) and GAPDH (Abcam, ab199554) primary antibodies (1:100) at 4 °C, and the addition of a fluorescently-labeled secondary antibody (1:100, Abcam, ab150077) the next day. After DPAI staining for 15 min, they were dried, mounted, observed, and photographed under a fluorescent microscope.

2.11. Animal samples

Twenty-four SPF C57BL/6 female mice, aged 6–8 weeks and weighing 20–25 g, were ordered from Jiangsu Recbio Technology Co., Ltd. (Animal Use License Number: SYXK (Su) 2021-0062). They were kept in an environment with appropriate temperature (20–26 °C), humidity (40–70 %), an alternating 12 h/12 h day/night cycle, and free eating and drinking.

2.12. Establishment of tumor-bearing mice

After adaptive feeding for one week, TU686 cells were adjusted to 1×10^7 cells/mL and injected into the subcutaneous tissue of armpit of mice (0.5 mL/mouse) to establish an LC tumor-bearing mouse model.

2.13. Mouse intervention

Two weeks later, the animals were randomized into 6 groups (n = 4). Of them, one group was not treated as a blank group, four groups were given intraperitoneal injection of DHA at concentrations of 20, 40, 80, and 160 μmol/L and labeled as groups A, B, C, and D, respectively, and the last group was given the same amount of DMSO solution by gavage and set as a control group. Three weeks later, all mice were sacrificed under anesthesia with their necks severed, and the intact subcutaneous tumor tissue was separated, weighed, and calculated. In addition, PCR, WB and IHC were performed to determine POSTN, YAP and IL-6 expression in tumor tissues with the same methods as mentioned above.

2.14. Statistical methods

The data, which were obtained after three repeated measurements for each experiment, were expressed by ($\bar{\chi}\pm s$). Results were analyzed using SPSS25.0 with a significance threshold of *P*-value <0.05, and the between-group and intra-group comparison methods used independent sample t tests and analysis of variance plus LSD tests, respectively.

3. Results

3.1. POSTN, YAP, and IL-6 expression in LC tissue

Quantitative PCR results showed that POSTN, YAP, and IL-6 mRNA levels in cancerous tissues were (2.60 ± 0.49), (2.42 ± 0.43) and (3.69 ± 0.47), respectively, all of which were higher compared with adjacent counterparts ($P < 0.05$, Fig. 1a). Similarly, WB identified elevated POSTN, YAP and IL-6 protein expression in cancerous tissues versus adjacent counterparts ($P < 0.05$, Fig. 1b). Finally, the positive expression of POSTN, YAP, and IL-6 was significantly higher in cancer tissues than in paracancerous tissues, according to IHC results (Fig. 1c). These results suggest that POSTN, YAP, and IL-6 are abnormally elevated in LC.

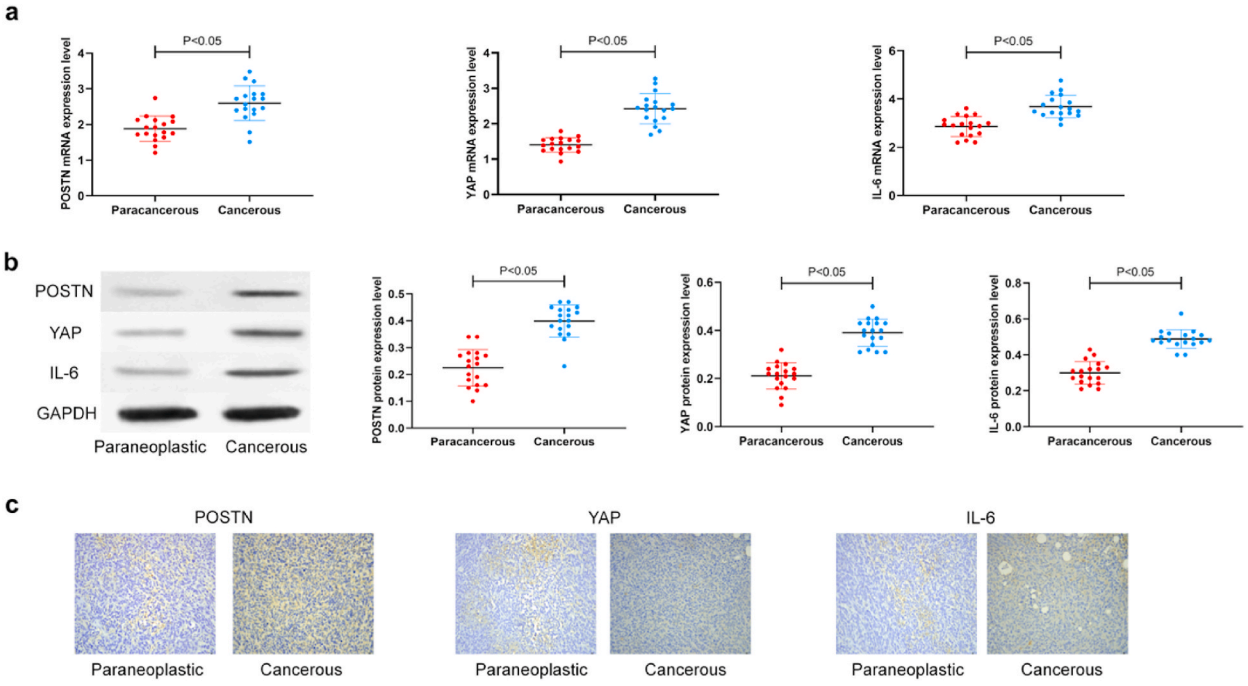

Fig. 1. POSTN, YAP, and IL-6 expression in LC tissue. a) PCR was performed to detect POSTN, YAP, and IL-6 mRNA expression. b) WB assay for POSTN, YAP, and IL-6 protein expression. c) IHC assays for POSTN, YAP, and IL-6 positive rates (200×).

3.2. Influence of DHA on biological behavior of LC cells

The biological behavior test revealed a lower cell cloning rate and a higher apoptosis rate of the four groups treated with DHA compared with the control group ($P < 0.05$). Similarly, the cell cloning rate was the lowest in group D and highest in group A among groups A, B, C, and D ($P < 0.05$, Fig. 2a). In terms of apoptosis, it was the highest in group D and the lowest in group A among the four DHA-intervened groups ($P < 0.05$, Fig. 2b). This means that DHA inhibits the proliferation of LC cells and promotes apoptosis.

3.3. Effects of DHA on the expression levels of POSTN, YAP and IL-6 in LC cells

Groups A, B, C, and D treated with DHA showed reduced POSTN, YAP and IL-6 levels than the control group after treatment ($P < 0.05$). According to PCR, POSTN, YAP and IL-6 mRNA levels were the lowest in group D, followed in ascending order by groups C, B, and A ($P < 0.05$, Fig. 3a). And as indicated by WB, groups C and D had similar POSTN, YAP and IL-6 protein expression ($P > 0.05$), lower than that in groups B and A ($P < 0.05$); while group A had higher POSTN, YAP and IL-6 than group B ($P < 0.05$, Fig. 3b). Finally, fluorescent staining results identified that the fluorescence intensity of POSTN, YAP, and IL-6 in group D was the lowest, while that in group A was the highest ($P < 0.05$, Fig. 3c). That is, DHA inhibited the expression of POSTN, YAP, and IL-6 in LC.

3.4. Effect of DHA on living tumors

The measurement of the subcutaneous intact tumor of six groups of mice showed that there was no significant difference in tumor volume and weight between the control and blank groups ($P > 0.05$), higher than those in the four groups of mice treated by DHA ($P < 0.05$); the tumor volume and weight of the four groups treated by DHA were group A, B, C, and D in descending order ($P < 0.05$, Fig. 4a–c). Finally, POSTN, YAP, and IL-6 levels in tumor tissues of the 6 groups were detected. Their levels were similar in the blank and control groups ($P > 0.05$), but were decreased in the other four groups of mice ($P < 0.05$). Among them, group D showed the lowest mRNA and protein expression of POSTN, YAP and IL-6 and group A showed the highest ($P < 0.05$, Fig. 4c). Similarly, the IHC results showed that the positive expressions of POSTN, YAP, and IL-6 in group D mice were significantly decreased ($P < 0.05$, Fig. 4d). These results are consistent with the results of the cell assay above, confirming the inhibitory effect of DHA on LC, as well as inhibiting the expression of POSTN, YAP and IL-6.

4. Discussion

In the present study, we found that POSTN/YAP/IL-6 was abnormally highly expressed in LC, and the use of DHA inhibited the growth of LC cells by a mechanism that may be related to the inhibition of the expression of POSTN/YAP/IL-6.

From the above, we believe that the influence of DHA on LC may be related to the POSTN/YAP/IL-6 pathway. To testify this claim, we first measured POSTN, YAP, and IL-6 levels in LC and adjacent tissue specimens, and identified their elevated expression in cancerous tissues, suggesting the high expression of the three in LC, which is consistent with the results of previous related studies [15–17] and can support our experimental results. Zhu M et al. have reported the important influence of POSTN on the EMT of LC cells and the ability of high POSTN expression to promote the malignant invasion and metastasis of LC cells [18]. This is similar to our findings and suggests that POSTN plays an oncogenic role in LC. Similarly, Tang X et al. found that VASN promoted LC proliferation

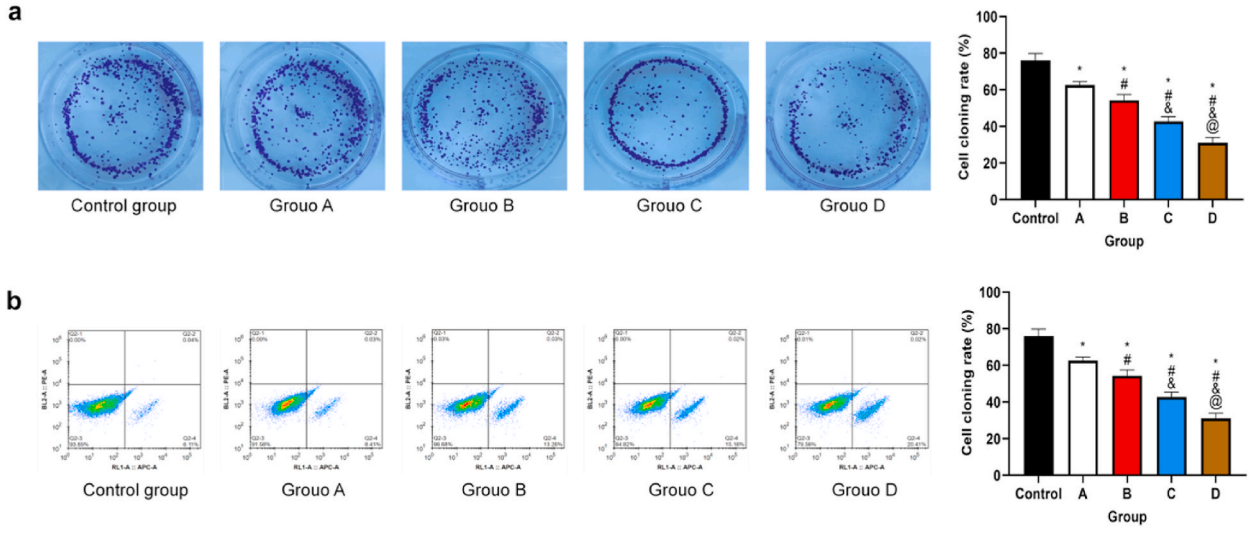

Fig. 2. Influence of DHA on biological behavior of LC cells. a) Results of cell cloning experiments. b) Flow cytometry detection of apoptosis rate. Note: *, #, & and @ indicate statistically significant differences from control group, group A, group B, and group C, respectively ($P < 0.05$).

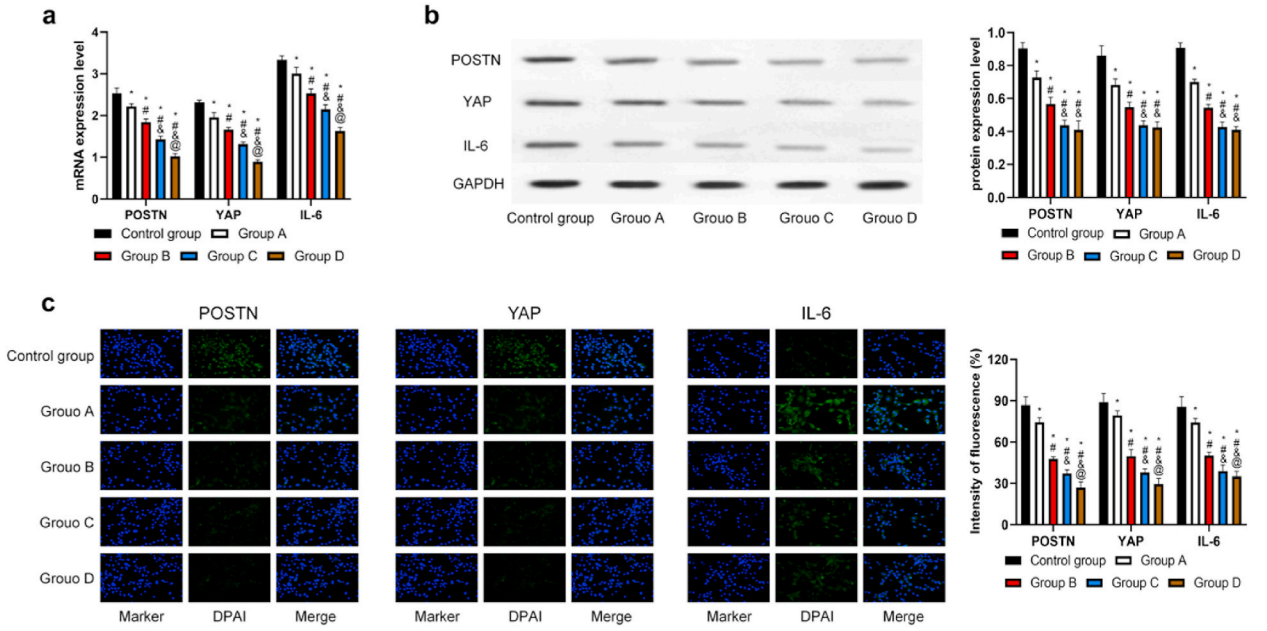

Fig. 3. Impacts of DHA on POSTN, YAP and IL-6 in LC cells. a) PCR was performed to detect POSTN, YAP, and IL-6 mRNA expression. b) WB assay for POSTN, YAP, and IL-6 protein expression. c) Fluorescent staining was performed to detect the fluorescence intensity of POSTN, YAP, and IL-6 (200×). Note: *, #, & and @ indicate statistically significant differences from control group, group A, group B, and group C, respectively ($P < 0.05$).

through YAP [19], further demonstrating the important influence of YAP on LC. In addition, Tsinias G et al. believed that high YAP expression predicted an increased risk of prognostic death in LC patients [20], which also suggests that YAP can be an objective clinical indicator to evaluate LC progression in the future. This has important implications for LC whose incidence is increasing and therapeutic targets are lacking. However, since only 4 clinical cases were included in the study of Tsinias G et al., the prognostic analysis results may be highly contingent. In this study, the prognostic significance of YAP in LC could not be verified due to the short research period and the absence of prognostic follow-up. Finally, it is well known that IL-6, a member of the IL family, is also a classic inflammatory factor in clinical practice. In many tumor diseases, including LC, the role of IL-6 has been repeatedly verified [21,22], so the results of this experiment can be expected. Combined with previous studies, we believe that POSTN can activate YAP signal transduction in LC and produce excessive IL-6, thus promoting the progression of LC [14].

Statistics show that the 5-year survival rate of patients with advanced LC is only about 15–20%, so the search for more effective LC diagnosis and treatment schemes is still the key project of clinical research [23,24]. Artemisinin is a widely recognized and applied antimalarial drug. DHA, its first-generation derivative with good water solubility and low toxicity, has been increasingly concerned due to its anticancer effects [25]. Fully understanding and mastering the anti-cancer action of DHA against LC may provide new research directions for future LC therapies. DHA has been preliminarily confirmed to be effective in inhibiting the progression of LC [26], but the specific mechanism needs further validation. Therefore, we observed the influence of DHA on LC cells through in vitro experiments. After DHA intervention, the proliferation ability of LC cells was obviously reduced, while the apoptosis rate was increased, consistent with the previous research results [27]. Importantly, the biological behavior changes of LC cells treated with 160 μmol/L DHA were the most significant, preliminarily indicating that increasing the dose of DHA can kill LC cells more effectively, which lays a foundation for a new treatment plan for LC in the future. Moreover, in the nude mice tumorigenesis experiment, the tumor growth of DHA-treated mice was more obviously inhibited, which can also support our view.

Finally, DHA intervention led to significantly decreased POSTN, YAP, and IL-6 levels in LC cells and living tumors, confirming that DHA can inhibit POSTN/YAP/IL-6 activation in LC, which is in line with previous studies. In relevant research on DHA, researchers agree that its anticancer effect mainly lies in Ref. [28] the fact that DHA can reverse the EMT progress of tumor cells [29,30]. As we all know, POSTN/YAP/IL-6 is a signal transduction pathway closely related to EMT [31,32]. The relationship between POSTN/YAP/IL-6 and EMT in LC cells was also mentioned above, so we hold that the effect of DHA on POSTN/YAP/IL-6 may also be related to the regulation of EMT in LC. However, due to limited experimental conditions, we have not yet detected EMT marker proteins after DHA intervention in this study, warranting more experiments to verify this idea.

In addition, further construction of the abnormal expression vectors of POSTN and YAP is needed to analyze the biological behavior changes of LC cells. Moreover, it is necessary to confirm the influence of DHA on LC growth in vivo, so as to realize the clinical application of DHA.

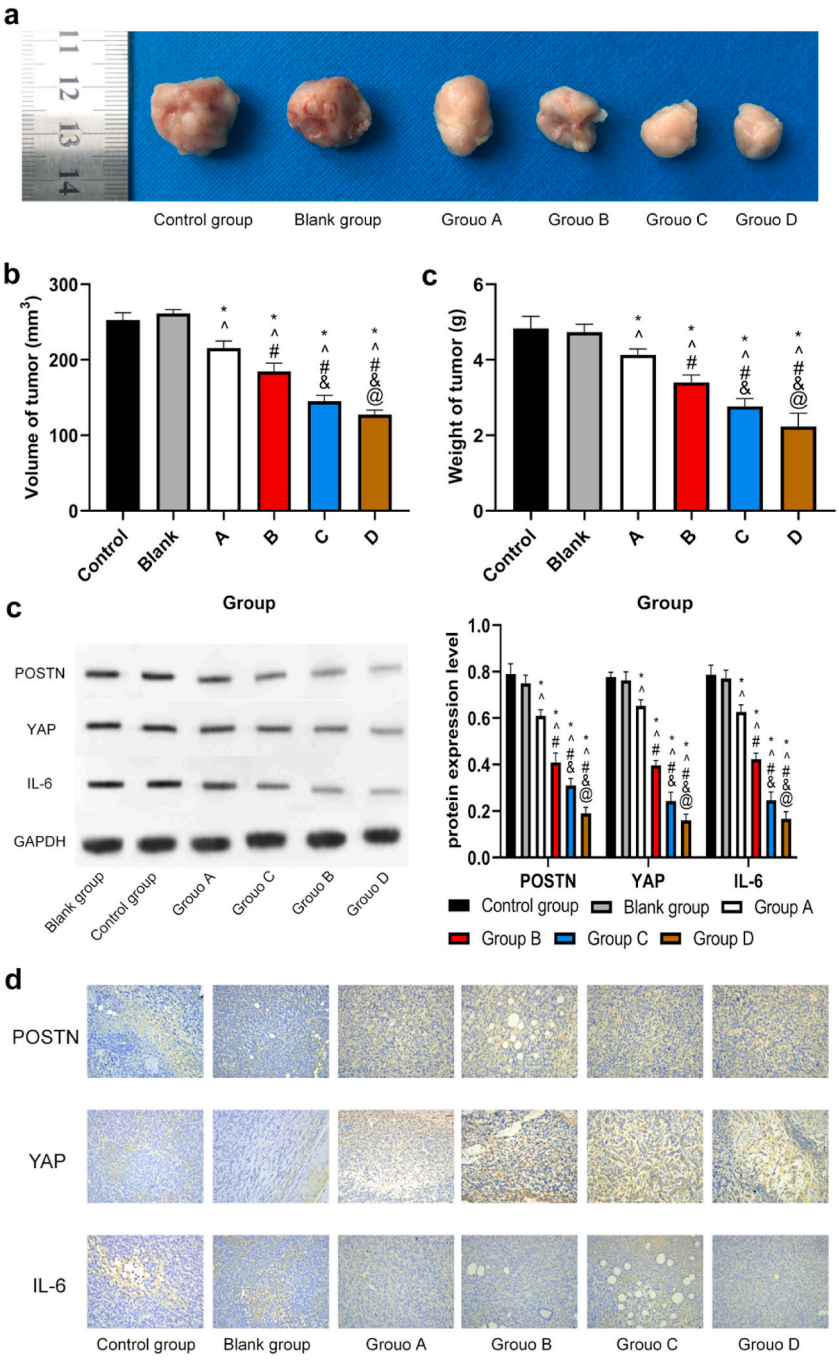

Fig. 4. Effect of DHA on living tumors a) The intact tumor. b) The volume of the tumor. c) The weight of the tumor. d) WB assay for POSTN, YAP, and IL-6 protein expression. e) IHC assays for POSTN, YAP, and IL-6 positive rates (200×). Note: *, ˆ, #, & and @ indicate statistically significant differences from control group, blank group, group A, group B, and group C, respectively ($P < 0.05$).

5. Conclusion

POSTN/YAP/IL-6 is highly expressed in LC and the use of DHA can inhibit the transduction of POSTN/YAP/IL-6, accelerating the apoptosis of LC cells and alleviating the malignant progression of LC. The POSTN/YAP/IL-6 pathway is expected to become a new treatment scheme for LC in the future, providing a more reliable guarantee for the prognosis, health, and safety of LC patients.

Ethical approval

The study protocol was approved by the Ethics Committee of Baoding No.1 Central Hospital(Approval No:2023003).

Consent to publish

All authors gave final approval of the version to be published.

Availability of data and materials

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

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CRediT authorship contribution statement

Xin-yu Zhang: Writing – review & editing, Writing – original draft. **Rui-cong Li:** Formal analysis, Data curation. **Cong Xu:** Supervision, Methodology. **Xiao-ming Li:** Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Not applicable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e27494.

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