Abstract. Postoperative recurrence causes a high mortality rate among patients with hepatocellular carcinoma (HCC). The current study aimed to determine the effects of Plasmodium infection on HCC metastasis and recurrence. The antitumor effects of Plasmodium infection were determined using two murine orthotopic HCC models: the non-resection model and the resection model. Tumour tissues derived from tumour-bearing mice treated with or without Plasmodium infection were harvested 15 days post-tumour inoculation. The expression levels of biomarkers related to epithelial-mesenchymal transition (EMT) and molecules associated with CC-chemokine receptor 10 (CCR10)-mediated PI3K/Akt/GSK-3β/Snail signalling were identified using reverse transcription-quantitative PCR and western blotting. The results demonstrated that Plasmodium infection significantly suppressed the progression, recurrence and metastasis of HCC in the two mouse models. The expression levels of E-cadherin were significantly higher in the Plasmodium-treated group compared with that in the control group, whereas the expression levels of Vimentin and Snail were significantly lower in the Plasmodium-treated group. Furthermore, Plasmodium infection inhibited the activation of Akt and GSK-3β in the tumour tissues by downregulating the expression levels of CCR10 and subsequently suppressing the accumulation of Snail, which may contribute to the suppression of EMT and the prevention of tumour recurrence and metastasis. In conclusion, the results of the present study demonstrated that Plasmodium infection inhibited the recurrence and metastasis and improved the prognosis of HCC by suppressing CCR10-mediated PI3K/Akt/GSK-3β/Snail signalling and preventing the EMT. These results may be important for the development of novel therapies for HCC recurrence and metastasis, especially for patients in the perioperative period.

Introduction

The annual incidence of liver cancer increased by 75% between 1990 and 2015 worldwide (1). Hepatocellular carcinoma (HCC) comprise ~75-85% of liver cancers (2), which constitute a huge health burden, especially in Asia where 72% of cases occur (3). Surgical resection is one of the main treatment methods for HCC; however, it cannot prevent post-operative recurrence (4). The HCC recurrence rate at 2 years post-surgery is 61.6% according to the statistics provided
by the World Health Organization (2018) (2). At present, the metastasis of liver cancer cells cannot be prevented by surgical resection, and there are a lack of effective drugs for the prevention and treatment of metastasis (5).

The epithelial-mesenchymal transition (EMT) induces cancer metastasis by promoting the separation and invasion of cancer cells from homologous cells (6). E-cadherin has been reported to maintain the tight connection between cells and prevent cell invasion and metastasis, and the decrease in the levels of E-cadherin is a common EMT biomarker (7). The upregulation of Vimentin and the transcription factor Snail are also commonly used EMT biomarkers (8,9). Recent studies have emphasized the vital role of EMT in the aggressive progression of HCC (10-13), in this regard further efforts for identification of EMT regulatory mechanisms will facilitate the development of novel strategies to combat metastasis. Chemokines and chemokine receptors serve key roles in the invasion and metastasis of malignant tumours (14-16). CC-chemokine receptor 10 (CCR10) has been demonstrated to be significantly upregulated in HCC specimens compared with the normal liver specimens (10). Furthermore, PI3K/Akt/GSK-3β has been demonstrated to be a classical signalling pathway via which CCR10 activates phosphorylation of Akt (16).

*Plasmodium* is a genus of single-cell protozoa that causes malaria, which may enter hepatocytes for dormancy or reproduction and lead to cell rupture, release merozoites and invade red blood cells (17). Although *Plasmodium* stays in the liver and causes black colouring of the tissue due to the deposition of the *Plasmodium* pigment, it does not damage liver function (18). Our previous studies have demonstrated that *Plasmodium* infection inhibits tumour development and metastasis in a murine Lewis lung cancer model (19-24). However, although *Plasmodium* infection has been demonstrated to suppress HCC angiogenesis by decreasing the infiltration of tumour-associated macrophages and reducing their matrix metalloproteinase 9 levels (25), the effects of *Plasmodium* infection on HCC metastasis and recurrence remain unclear.

To gain further insight into the effects of *Plasmodium* infection on HCC metastasis and recurrence, the antitumor effects of *Plasmodium* infection using two murine orthotopic models were studied, with an emphasis on the regulation of *Plasmodium* infection on EMT of HCC. Here, it was demonstrated that *Plasmodium* infection prevented recurrence and metastasis of HCC potentially via CCR10-mediated PI3K/Akt/GSK-3β/Snail signalling.

**Materials and methods**

**Ethics statement.** Ethical approval was provided by Guangzhou Institute of Biomedicine and Health, Chinese Academy of Sciences (approval no. N2019014; Guangzhou, China). All animal experiments in this study were performed following the standard guidelines for the care of animals, which were approved by the Welfare Committee of the Center of Experimental Animals. According to the international regulations, the mice were euthanised when the tumour diameter reached 2 cm. All efforts were made to minimise animal suffering.

**Antibodies.** Western blotting was performed using the following primary antibodies: GAPDH (cat. no. ab9385; Abcam), E-cadherin (cat. no. BS1098; Bioworld Technology, Inc.), Snail (cat. no. 3879S; Cell Signaling Technology, Inc.), PI3K (cat. no. ab151549; Abcam), phosphorylated (p)-PI3K p85 (Tyr458)/p55 (Tyr199) (cat. no. 4228S), Akt (cat. no. 9272S), p-Akt (Ser473) (cat. no. 4060S), GSK-3β (cat. no. 12456S), p-GSK-3β (Ser9) (cat. no. 5585S) (all Cell Signaling Technology, Inc.) and CCR10 (cat. no. ab3904; Abcam).

**Animals, cells and parasites.** Female C57BL/6 mice (age, 6-8 weeks) purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. were bred in the Animal Center of Guangzhou Institute of Biomedicine and Health (GIBH; Guangzhou, China) according to the Guide to the Care and Use of Laboratory Animal Committee of the Institute. Mice were housed under specific pathogen-free conditions with a 12-h light/dark cycle and were provided food and water *ad libitum*. The Hepa1-6 luciferase liver cancer cell line derived from C57BL/6 mice was obtained from The First Affiliated Hospital of Sun Yat-Sen University and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% foetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), and was incubated in a humidified atmosphere with 5% CO₂ at 37°C. The *Plasmodium yoelii* nonlethal strain (Py17XLN) was obtained from the Malaria Research and Reference Reagent Resource Center (MR4).

**Animal models.** Mice undergoing surgery were anaesthetised by intraperitoneal injection of 240 mg/kg 1.25% Avertin. Two animal models were established. The tumour volumes were calculated as follows: Volume=(length x width²)/2. Mice were euthanized by exsanguination under anaesthesia with 240 mg/kg Avertin.

**Non-resection model.** The non-resection mouse model was established by intrahepatic injection of 5x10⁵ Hepa1-6 luciferase cells into the left liver lobe. Female C57BL/6 mice (n=56) were randomly divided into two groups (n=28 mice/group). The control group (Hep) received 5x10⁵ Hepa1-6 luciferase cells only. The mice in the *Plasmodium*-infected group (Hep + Py) were orthotopically implanted with 5x10⁵ Hepa1-6 luciferase cells and intraperitoneally injected with 5x10⁵ *Plasmodium yoelii* 17XLN-parasitised erythrocytes. Subsequently, 26 mice (n=13 mice/group) were randomly selected for observation of survival, dynamics of parasitaemia and weight changes. The tumours in the remaining 30 mice (n=15 mice/group) were harvested on day 15 post-inoculation for further experiments.

**Resection model.** To investigate the effects of *Plasmodium* infection on postoperative recurrence, the resection mouse model was established by an intrahepatic injection of tumour pieces and tumour resection on day 21 post-implantation. Tumours were derived from subcutaneous tumour-bearing mice, which had been subcutaneously injected with 5x10⁵ Hepa1-6 luciferase cells. The tumours were harvested and cut into 1-mm³ pieces on day 14 post-inoculation. The tumour pieces were implanted into the livers (left liver lobe) of 20 mice,
and in vivo luciferase imaging was used to monitor the survival of the transplanted tumours once a week in the following days. The mice underwent resection on day 21 post-inoculation to completely remove the transplanted tumour mass and were randomly divided into two groups according to the inoculation with or without Plasmodium on day 3 post-surgery. The primary outcomes were death and intraperitoneal metastasis caused by HCC in both groups.

In vivo luciferase imaging. To evaluate tumour survival, the luminescence properties of luciferase were used to track HCC in vivo. Each mouse was intraperitoneally injected with 10 µl/g potassium fluorescein as a substrate. The IVIS Spectrum (PerkinElmer, Inc.) in vivo imaging system was used to monitor the fluorescence emitted by Hepa1-6 luciferase at 10-20 min post-injection.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from tumour tissues using TRIzol® reagent (cat. no. 15596018; Invitrogen; Thermo Fisher Scientific, Inc.) and reverse-transcribed (37°C for 15 min, 85°C for 5 sec and 4°C hold) using a cDNA Synthesis kit (cat. No. RR047A; Takara Bio, Inc.). qPCR was performed using TB Green Premix Ex Taq (cat. no. RR820A; Takara Bio, Inc.) with CFX96 Touch PCR machine (Bio-Rad Laboratories, Inc.). The qPCR procedures were as follows: Predenaturation at 95°C for 30 sec; denaturation at 95°C for 5 sec, annealing and extension at 60°C for 30 sec (39 cycles); denaturation at 95°C for 10 sec; and finally melt curve at 65-95°C for 5 sec. The mRNA levels of the genes of interest were normalised to those of β-actin. The primer sequences used were as follows: β-Actin forward, 5'-TCTGGCACCCACCTCTCAGC-3' and reverse, 5'-TCA CTCTTTCACCGGTGGCCT-3'; Vimtinen forward, 5'-CTG CGAGAGAAATTGCAGGAG-3' and reverse, 5'-CTTCTA TACTGCTGGCGCAC-3'; CCR10 forward, 5'-CAAGGCCCA CAGACGAGGTC-3' and reverse, 5'-GATCGGATGATGTT CGCTTGCC-3'. The 2^ΔΔCq method was used to quantify the relative changes in gene expression (26). Each sample was tested in at least three independent replicates.

Protein extraction and western blotting. Tissues were lysed in RIPA lysis buffer (cat. no. KGP702-100; Nanjing KeyGen Biotech Co., Ltd.) containing 1% phenylmethanesulphonyl fluoride (Beyotime Institute of Biotechnology) and phosphatase inhibitor cocktail (cat. no. HY-K0021; MedChem Express) on ice for 30 min and subsequently centrifuged at 16,000 x g for 15 min at 4°C to collect the supernatants. After protein determination using a BCA assay, protein samples containing 15-20 µg protein were separated using 10% polyacrylamide gel electrophoresis (SurePAGE; cat. no. M00665; GenScript) and transferred to polyvinylidene difluoride membranes (cat. no. ISEQ00010; EMD Millipore). The membrane was probed with anti-E-cadherin (1:500), anti-Snail1 (1:1,000), anti-p-PI3K (1:1,000), anti-PI3K (1:1,000), anti-p-AktSer473 (1:500), anti-Akt (1:300), anti-p-GSK3βSer9 (1:500), anti-GSK3β (1:500) and anti-β-actin (1:2,000) antibodies at room temperature for 2 h. Then, membranes were washed three times, 5 min each, with Tris-buffered saline with 0.1% Tween-20 (TBST). After incubation with HRP-conjugated Goat anti-Mouse IgG (cat. no. ab205718; Abcam) or HRP-conjugated Goat anti-Rabbit IgG (cat. no. ab205718; Abcam) at room temperature for 1 h (1:5,000), membranes were washed four times, 5 min each, with TBST. Then, the bands were detected using ECL reagents (cat. no. WBULS0500; EMD Millipore). Densitometry of band signals was quantified using Quantity One software (version 4.6.6; Bio-Rad Laboratories, Inc.). Data are presented as the ratio of the target protein to GAPDH densitometry values.

Giemsastain. Giemsastain was performed for determination of the ratio of Plasmodium-infected RBC in the blood. A small drop of mouse tail blood was collected on a glass slide to make a thin layer of blood smear. Then, using a Pasteur pipette, the thin film was fixed by carefully dropping methanol onto the thin film. The slides were placed on a drying rack on a flat surface for ~2 min to let the blood film dry completely. Then, Giemsa working solution (cat. no. 48900; Sigma-Aldrich; Merck KGaA) was poured slowly on the slide until the blood films were covered. After 40 min of staining, the stain was washed from the slides and then the slides were allowed to air-dry. Finally, the ratio of parasitaemia was calculated using the following formula: Parasitaemia (％)=(the number of Plasmodium-infected RBC per 1,000 RBC/1,000) x100%.

Statistical analysis. Statistical analysis was performed using GraphPad Prism 8.0 (GraphPad Software, Inc.) and the R software (version 3.6.3; http://www.R-project.org) (27). Data of normally distributed variables are presented as the mean ± standard deviation. Categorical data are presented as frequency and percentages. An independent sample Student's t-test was used to compare the means of two groups. The Fisher's exact test was used to compare the rate of metastasis between two groups. Overall survival (OS) in the two groups was estimated using the Kaplan-Meier method, and the OS differences between the two groups were compared using the log-rank test. P<0.05 was considered to indicate a statistically significant difference. All of above experiments were replicated at least twice and the results were similar.

Results

Plasmodium infection significantly inhibits HCC progression and metastasis in a non-resection mouse model. In this experiment, luciferase imaging results revealed that Hepa1-6 cells were successfully implanted into all mice, and the fluorescence signal was significantly lower in Hep + Py group compared with that in the Hep group on day 15-post inoculation (Fig. 1A). The peak of infection appeared on ~day 18 post-intraperitoneal injection of the parasite in both models; the highest Plasmodium density reached on average 55-60% and decreased to ~5% on day 24 (Figs. S1A and B and S2). There was no significant difference in body weight between the two groups during the course of Plasmodium infection (in the treated group), whereas the weight was not comparable between the two groups thereafter since the majority of the mice in the control group died (Fig. 1B). Notably, the tumour-bearing mice treated with Plasmodium exhibited a higher survival rate (53.8%, 7/13) and a longer OS time (until to the end of the study) compared with that observed in the control group (median interval, 21 days) (Fig. 1C). The tumour volume on day 15 post-inoculation in the Hep + Py group
Plasmodium infection inhibits EMT and prevents recurrence of liver cancer. The resection mouse model was established by intrahepatic implantation of tumour pieces, followed by complete tumour resection on day 21 post-implantation (Fig. 2A). In the early stage, no significant differences were observed in the body weight between the mice in the two groups.

Following tumour resection, weight loss and short-term weight recovery occurred in the Hep + Py group. During the period of infection, the food intake of mice in the Plasmodium infection group was poor; thus, the weight gain in the Plasmodium infection group was slower compared with that in the control group. Following self-healing from infection, the weight in the Plasmodium infection group continued to increase, whereas that of the control group decreased due to tumour development (Fig. 2B). The effect of surgical resection followed by Plasmodium infection on weight loss was notable when comparing the infected group in the resection model with the infected group in the non-resection model (Figs. 1B and 2B). A phenomenon of hemozoin accumulation (dark colour) (28) was observed in the liver in the Plasmodium-infected group (Fig. 2D) and the non-resection (Fig. 1E) models. Notably, the results demonstrated that the Plasmodium-treated
tumour-bearing mice survived longer compared with the control mice (Fig. 2C). The cumulative recurrence rate in the *Plasmodium* infection group (20%, 2/10) was significantly lower compared with that in the control group (80%, 8/10) on day 75 post-inoculation (Fig. 2D).

**Plasmodium infection inhibits HCC metastasis possibly through EMT suppression.** E-cadherin is the key cadherin that serves an antimetastatic role and is the most important EMT biomarker (6). In the present study, the expression levels of E-cadherin in the Hep + Py group were significantly higher compared with those in the Hep group (Fig. 3A). As one of the biomolecules that binds to the E-boxes in the E-cadherin promoter, Snail directly downregulates the expression of E-cadherin to promote the occurrence of EMT (29). The results of the present study demonstrated that the expression levels of Snail were significantly lower in the Hep + Py group compared with those in the Hep group (Fig. 3A). The RT-qPCR results showed that Vimentin expression, one of the molecular markers of EMT, was significantly decreased in the Hep + Py group compared with in the Hep group (Fig. 3B).

**Plasmodium infection suppresses the activation of the PI3K/Akt/GSK-3β signalling pathway.** Previous studies have reported that the PI3K/Akt/GSK-3β signalling pathway and the transcriptional repressor Snail are required for EMT in HCC (14,16). Therefore, the effects of *Plasmodium* infection on the PI3K/Akt/GSK-3β signalling pathway was tested in HCC cells. Western blot analysis demonstrated that the phosphorylation of Akt (Ser473) (Fig. 4A and C) and GSK-3β (Ser9) (Fig. 4A and D) was downregulated in the *Plasmodium*-treated group compared with that in the control group, although the difference in PI3K phosphorylation levels between the two groups was not statistically significant (Fig. 4B).

**Plasmodium infection downregulates the expression of CCR10.** The chemokine/chemokine receptor axis has been demonstrated to activate the PI3K/Akt/GSK-3β/Snail signaling pathway in HCC cells (14,16). As expected, the mRNA expression levels of CCR10 were significantly lower in the *Plasmodium*-treated group compared with those in the control group (Fig. 5B). Western blotting results revealed that the protein expression levels of CCR10 were also significantly lower in the infected group compared with those in the control group (Fig. 5A).

**Discussion**

Primary HCC accounts for 75-85% of liver cancer (8.2% of mortality rates in 2018), which is the fourth most common cause of cancer-related death worldwide (2,30). Recurrence is the leading cause of postoperative death in patients with liver cancer (31). The results of the present study demonstrated that *Plasmodium* infection significantly inhibited tumour growth...
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Figure 3. Plasmodium infection downregulates the expression levels of Snail and Vimentin and upregulates the expression levels of E-cadherin. (A) Western blot analysis of the levels of E-cadherin and Snail in tumour tissues of the Hep and Hep + Py groups. (B) Reverse transcription-quantitative PCR results of Vimentin expression showed that the level of mRNA in Hep + Py was significantly decreased compared with in the Hep group. Data are presented as the mean ± SD. **P<0.01, ***P<0.001. Hep, mice inoculated with Hepa1-6 cells; Py, Plasmodium yoelii 17XNL.

Figure 4. Plasmodium infection suppresses the activation of the PI3K/Akt/GSK-3β signalling pathway. (A) Western blot analysis of PI3K, p-PI3K, Akt, p-Akt, GSK-3β and p-GSK-3β in the Hep and Hep + Py groups. The ratio of (B) p-PI3K/PI3K, (C) p-Akt/Akt and (D) p-GSK-3β/GSK-3β in the two groups. Data are presented as the mean ± SD. *P<0.05. Hep, mice inoculated with Hepa1-6 cells; Py, Plasmodium yoelii 17XNL; p-, phosphorylated.
and abdominal metastasis in two mouse models, including a murine HCC resection model. The underlying mechanisms of action may involve the inhibition of the PI3K/Akt/GSK-3β/Snail signalling pathway by downregulating the expression levels of CCR10 and p-Akt, but not PI3K; the downregulation of p-GSK-3β, but not GSK-3β; the downregulation of Snail, but not p-Snail; and the upregulation of E-cadherin. Notably, the downregulation of Snail and the upregulation of E-cadherin may contribute to the suppression of the EMT in murine HCC resection and non-resection models. HCC, hepatocellular carcinoma; CCR10, CC-chemokine receptor 10; Py, Plasmodium yoelii 17XNL; p-, phosphorylated; EMT, epithelial-mesenchymal transition; CCL, C-C motif chemokine ligand.
CCR10, reducing the accumulation of Snail and upregulating the levels of E-cadherin.

The EMT has been implicated in carcinoma invasion and metastasis (32). Previous studies have suggested that the downregulation of E-cadherin is a prominent feature of the EMT. For example, the transcriptional activity and protein expression of E-cadherin has been reported to be enhanced by preventing the binding of the E-boxes of the EMT promoter to transcriptional barriers, including zinc-finger transcriptional repressors snail and slug, the repressor zinc-finger E-box-binding homeobox (ZEB)-2, ZEB-1, as well as the basic helix-loop-helix, transcription factors twist and E12/E47, which prevent the EMT (32,33). The results of the current study demonstrated that Plasmodium infection increased the expression levels of E-cadherin and downregulated the levels of Snail and Vimentin compared with those in the control group, which suggested that Plasmodium infection may prevent the EMT in HCC.

PI3K activates the secondary messenger PIP3, which activates Akt via phosphorylation at Ser308 (34). GSK-ββ is a downstream target of p-Akt (35), which promotes the degradation of Snail (36), whereas the phosphorylation of GSK-ββ at Ser9 by PI3K/Akt leads to its inactivation (37) and upregulation of Snail. Snail, which downregulates E-cadherin, is downstream to GSK-ββ; GSK-ββ inhibits the expression of Snail and increases the expression levels of E-cadherin (38). The results of the current study demonstrated that Plasmodium infection inhibited the phosphorylation of Akt, which subsequently inhibited the conversion of GSK-ββ to p-GSK-ββ and reduced the expression levels of Snail. Therefore, the inhibitory effects of Snail on E-cadherin expression were eliminated, and the EMT was prevented.

Chemokines exert their action through seven transmembrane spanning G-protein-linked chemokine receptors (39,40). Previous studies have suggested that chemokines and chemokine receptors are involved in inflammatory reactions and wound healing (41-43). A recent study has demonstrated that chemokines and their receptors serve a crucial role in tumour cell growth and metastasis in melanoma, lung cancer, gastric carcinoma, pancreatic cancer, colorectal carcinoma and HCC (44). CCR10 activation has been reported to stimulate the invasion and migration of HCC, breast cancer and melanoma cells (16,45,46). CCR10 and the downstream PI3K/Akt signalling pathway serve a role in HCC progression (16). The results of the present study demonstrated that Plasmodium infection downregulated the expression levels of CCR10 compared with those in the control group. The inhibition of CCR10-mediated PI3K/Akt/GSK-ββ/Snail signalling may result in the suppression of the EMT programming in HCC. However, one limitation of the present study was that experiments were only repeated twice.

In conclusion, the results of the current study identified a potential novel mechanism of Plasmodium infection against HCC in mice (Fig. 6), providing evidence for the prevention of recurrence and distant metastasis of HCC by regulating the EMT through infection with Plasmodium parasites. In addition, our previous studies have demonstrated that Plasmodium infection displays antitumor effects by activating the innate and acquired antitumor immunity, remodelling the tumour immnosuppressive microenvironment and inhibiting angiogenesis (20-25). Based on these studies, three clinical trials are ongoing in China (trial nos. NCT02786589, NCT03474822 and NCT04165590). These findings may be important for developing novel strategies for the treatment and prevention of metastasis and recurrence of HCC, especially for patients in the perioperative period.

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Availability of data and materials

All data generated or analysed during this study are included in this published article. The manuscript has been published as a preprint.

Authors’ contributions

XPC, XWZ, YL, XC and LQ conceived and designed the study. YL, XC, MM and LQ completed in vitro culture experiments and performed the in vivo operations. ZT, WTD and LLD assisted in the in vivo operations and conducted the staining experiments. YL, MM, DA and SF carried out the western blotting and PCR assays. YL, MM, XC, STZ and XFL performed the data analysis and interpretation. XFL, XZW, XPC and LQ made substantial contributions to the writing of the manuscript. All authors agreed to be accountable for all aspects of the work. YL, XC, XPC and XWZ confirmed the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The specific procedures used during the animal study were approved by the Ethics Committee of The Animal Laboratory Center of Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, and obtained the corresponding IACUC (approval no. N2019014). All efforts were made to minimise animal suffering.
Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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