Plasmodium infection prevents recurrence and metastasis of hepatocellular carcinoma potentially via inhibiting EMT

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Primary research

Keywords: Plasmodium, Hepatocellular Carcinoma, Metastasis, EMT

Posted Date: May 28th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-29656/v1>

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Abstract

Background: Hepatocellular carcinoma (HCC) is the fourth leading cause of cancer mortality worldwide and is characterized by a high rate of recurrence. We have previously reported *Plasmodium* infection inhibits tumor development and metastasis in a murine Lewis lung cancer model. In the current study, we aimed to examine the effects of *Plasmodium* infection on HCC metastasis and recurrence.

Methods: Antitumor effects of *Plasmodium* infection were determined using two murine orthotopic HCC models, the non-resection model for investigating the effect of *Plasmodium* infection on liver tumor progression and metastasis, the resection model for investigating the effect of *Plasmodium* infection on the tumor recurrence after tumor was removed. Tumor tissues derived from tumor-bearing mice treated with or without *Plasmodium* infection were harvested after 15 days of tumor inoculation. The biomarkers related to epithelial-mesenchymal transition (EMT) and molecules associated with CCR10-mediated PI3K/Akt/GSK-3 β /Snail pathway signaling were identified by qRT-PCR and western blot.

Results: We found that *Plasmodium* infection significantly suppressed progression, recurrence and metastasis of HCC and prolonged the survival of tumor-bearing mice in both models. The expression levels of E-cadherin were significantly higher in *Plasmodium* treated group compared with those in control group, whereas the expression levels of Snail were significantly lower in the treated group than those in control group. Furthermore, *Plasmodium* infection inhibited the activation of Akt and GSK-3 β in the tumor tissues by downregulating the expression of CCR10, thereby suppressing the accumulation of Snail and potentially contributed to the suppression of EMT and the prevention of tumor recurrence and metastasis.

Conclusion: This study suggested that *Plasmodium* infection inhibited recurrence and metastasis, improved the prognosis of HCC potentially via suppression of CCR10-mediated PI3K/Akt/GSK-3 β /Snail signaling, and prevention of EMT. These findings may be important in the development of novel therapy for HCC recurrence and metastasis especially for patients during the perioperative period.

Introduction

Hepatocellular carcinoma (HCC) ranks fourth in the cause of cancer-related death worldwide. The annual incidence of this disease is believed to be on the rise, especially in Asia [1]. Surgical resection is still one of the main therapies for HCC, and the characteristics of early postoperative recurrence are indicative for the prognosis of this disease [2]. The HCC recurrence rate of 2 years after the operation is as high as 61.6%—which is the leading cause of cancer-associated death—according to the statistics of the World Health Organization [3]. Many factors correlate to the distant recurrence of liver cancer, for instance, serum AFP over 400ng/ml and tumor size larger than 5cm. Microvascular and portal vein cancer thrombus indicate distant metastasis of tumor cells that pass through vascular basement membrane after metamorphosis, and may contribute to HCC postoperative intrahepatic invasion in the future [4]. At

present, the metastasis of liver cancer cells cannot be completely blocked by surgical resection and effective drugs are lacking.

Epithelial-mesenchymal transition (EMT) is the process that epithelial cancer cells lose their polarity and change into motile mesenchymal cells, which is an important mechanism leading to distant metastasis of malignant tumors. EMT induces cancers metastasis through promoting the separation and invasion of cancer cells from homologous cells [5]. It has been demonstrated that E-cadherin can maintain the tight connection between cells and prevent cell invasion and metastasis, and the decrease of E-cadherin is a common EMT biomarker [6]. The upregulation of transcription factor Snail can also be used as a common biomarker of EMT [7, 8].

Evidence suggests that chemokines/chemokine receptors play key roles in the invasion and metastasis of malignant tumors [9-11]. Chemokines act as prominent recruiters in the invasive process of cancer cells. Through the effects of chemokines, tumor advantageous factors such as regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) accumulate around the tumor cells, therefore altering the microenvironment of tumors. One type of GPCR, CC-chemokine receptors 10 (CCR10), has been found to be significantly up-regulated in HCC tumors. CCR10 activates the phosphorylation of downstream signal molecules to promote EMT. Furthermore, PI3K/Akt/GSK-3 β has been demonstrated to be a classical signaling pathway for CCR10 to activate intracellular signaling molecules[11].

Our previous studies have demonstrated that *Plasmodium* infection significantly inhibits tumor growth and prolongs the survival time of lung cancer-bearing mice through activating the innate and adaptive anticancer immunity, remodeling the tumor immunosuppressive microenvironment, such as reducing the numbers of MDSCs and Tregs within tumor tissue, and inhibiting tumor angiogenesis [12-17]. However, the effects of *Plasmodium* infection on HCC metastasis and recurrence especially in tumor-resected animals remains unclear. Furthermore, there are no any data regarding the possible effects of *Plasmodium* infection on EMT pathways.

Here, we investigated the antitumor response of *Plasmodium* infection in progression, recurrence and metastasis of HCC in murine orthotopic resection model and non-resection model. We found that *Plasmodium* infection significantly inhibited growth, recurrence and metastasis of HCC and prolonged the survival of tumor-bearing mice in both models. Furthermore, significantly higher expression levels of E-cadherin and lower expression levels of Snail were observed in *Plasmodium* treated group compared with those in control group. Moreover, the activation of AKT and GSK-3 β and the expression of CCR10 were suppressed by the infection. These findings suggest that *Plasmodium* infection inhibits progression, recurrence and metastasis of HCC in tumor resection and non-resection models potentially via suppression of CCR10-mediated AKT/GSK-3 β /snail signaling, which provides a possible new mechanism of action for further research.

Materials And Methods

Ethics statement

The animal experiment facilities were approved by the Guangdong Provincial Department of Science and Technology and complied with the guidelines of the Animal Care Committee, Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences. According to the international regulations, the mice were killed in time within the legal tumor size. All efforts were made to minimize animal suffering.

Antibodies

Western blotting was conducted using the following primary antibodies: GAPDH (Abcam#ab9385), E-Cadherin (Bioworld#Cat#BS1098), Snail (CST, Cat#3879S), p-PI3K p85 (Tyr458)/p55 (Tyr199) (CST, Cat#4228S), Akt (CST, Cat#9272S), p-Akt (Ser473) (CST, Cat#4060S), GSK-3 β (CST, Cat#12456S), p-GSK-3 β (Ser9)(CST, Cat#5558S), CCR10 (Abcam, Cat#ab3904)

Sources of animals, cells, and parasites

Six to eight-week old female C57BL/6 mice purchased from Vital river Experiment Animal Limited Company (Beijing, China) were bred in the Animal Center of Guangzhou Institute of Biomedicine and Health#GIBH#following the Guide to the Care and Use of Laboratory Animal Committee of the institute. Mice were housed in specific pathogen-free (SPF) conditions, with a 12-hours light cycle and food and water at ad libitum. All animal experiments were performed with the standard guidelines for the care of animals, which were approved by the Welfare Committee of the Center of Experimental Animals (Guangzhou, China). The Hepa1-6 Luciferase cell line, a murine liver cancer cell line derived from C57BL/6 mice was obtained from our deposit at GIBH and was incubated in a humidified atmosphere of 5% CO₂ at 37°C. *Plasmodium yoelii* nonlethal strain (Py17XNL) was obtained from the Malaria Research and Reference Reagent Resource Center (MR4).

Animal models

To better understand the effect of *Plasmodium* infection on HCC recurrence and metastasis, two animal models were established as follows. Tumor volumes were calculated as follows: tumor volume = (length*width²)/2.

The non-resection model: To determine the effect of *Plasmodium* infection on HCC metastasis, the non-resection mouse model was established by intrahepatic injection of 5×10⁵ Hepa1-6 Luciferase cells. Female C57BL/6 mice (n=56) were randomly divided into two groups, with 28 in each group. Control group (Hep) were only seeded with 5×10⁵ Hepa1-6 Luciferase cells. *Plasmodium* infected group (Hep + Py) were orthotopically implanted with 5×10⁵ Hepa1-6 Luciferase cells and intraperitoneally injected with 5×10⁵ *Plasmodium yoelii* 17XNL parasitized erythrocytes at the same time. Then 26 mice were randomly selected (13 from each group) for observation of survival, dynamics of parasitemia, and weight changes. Tumors in the remaining 30 mice (15 from each group) were harvested 15 days post-inoculation for further experiments.

The resection model: To investigate the effect of *Plasmodium* infection on post-operation recurrence, the resection mouse model was established by intrahepatic injection of tumor pieces and undergoing tumor resection on day 21 after tumor implantation. Tumors were derived from subcutaneous tumor-bearing mice, which was established by subcutaneous injected with 5×10^5 Hepa1-6 Luciferase cells. Tumors were harvested and cut into 1mm^3 pieces on day 14 post inoculation. The tumor pieces were implanted into the livers of 20 mice and Luciferase in vivo imaging was used to monitor the surviving of transplanted tumors in the following days. These mice underwent reoperation on day 21 post inoculation to completely remove the transplanted tumor mass. Then the mice were randomly divided into 2 group according to the inoculation with or without the parasite on day 3 after surgery. Twenty mice (10 each group) were used for the observation of survival and intraperitoneal metastasis of hepatocellular carcinoma.

Luciferase in vivo imaging

To evaluate tumor survival, we used the luminescence properties of luciferase to track HCC in vivo. In the trial, each mouse was injected intraperitoneally with potassium fluorescein as a substrate according to body weight (10 ul/g fluorescein potassium each mouse). IVIS Spectrum was used to monitor the fluorescence emitted by Hepa1-6 Luciferase about 10 to 20 minutes later.

Quantitative real-time PCR

Total RNA of tumor tissues were extracted with TRIzol reagent (Invitrogen Cat#15596018) and the RNA was reverse transcribed with the use of cDNA synthesis kit (TAKARA Cat#RR047A). Quantitative real-time PCR reactions were performed using TB Green Premix Ex Taq (TAKARA Cat#RR820A). The mRNA levels of the genes of interest were normalized to that of *Actin*. The Primer sequences for RT-qPCR were as follows: *Actin*: forward 5'-TCTGGCACCACACCTTCTAC-3' and reverse 5'-TCATCTTTTCACGGTTGGCCT-3', *CCR10*: forward 5'-CAAGCCCACAGAGCAGGTCTC-3' and reverse 5'-GATCGGGTAGTTCGTCTGGC-3', each sample was tested at least three independent replicates.

Protein extraction and Western blotting

Tissues or cells were lysed in RIPA lysis buffer (KeyGene Cat#KGP702-100) containing 1% phenylmethanesulfonyl fluoride (Beyotime) and phosphatase inhibitor (Cocktail, Cat#HY-K0021) on ice for 30min, and then centrifuged to collect the supernatants. Protein sample with equal quantity was separated by PAGE electrophoresis (SurePAGE, Cat#M00665), and then transferred to PVDF membranes (Millipore, Cat#ISEQ00010). The membrane was probed with anti-E-cadherin (1:500), anti-Snail1 (1:1000), anti-p-AKT^{Ser473} (1:500), anti-AKT (1:300), anti-p-GSK3 β ^{Ser9} (1:500), anti-GSK3 β (1:500), anti- β -actin (1:2000). After incubation with an appropriate secondary antibody, bands were detected with ECL reagents (Millipore, Cat# WBULS0500). Densitometry of Band signals were quantified using Quantity One. Results were presented as the ratio of the target protein's densitometry units to GAPDH's densitometry units.

Statistical analysis

Statistical analysis was performed using GraphPad Prism. Results are presented as means $\pm$ standard deviations. A two independent sample t test was applied to compare means of two groups. One-way ANOVA was applied to compare means of multiple groups. Chi-square test was applied to compare the rate of metastasis of two groups. A P-value lower than 0.05 was considered statistically significant. P-value less than 0.05, 0.01, and 0.001 were indicated by *, **, and *** respectively, in each figure.

Results

***Plasmodium* infection significantly inhibited HCC progression and metastasis in the non-resection mouse model**

To investigate the effect of *Plasmodium* infection on the growth of HCC, mice were orthotopically seeded with Hepa1-6 luciferase cells and at the same time infected with *Plasmodium yoelli* 17XNL (Hep + Py) or uninfected with the parasite (Hep). In this experiment, Luciferase in vivo imaging was used to examine the viability of liver cancer cells, since the fluorescence could be released by Hepa1-6 luciferase cells once combined with the substrate. The imaging results showed that all mice were successfully implanted with Hepa1-6 cells and the fluorescence signal was significantly lower in *Plasmodium* treated group compared with that in the control group on day 15 post inoculation (Fig.1A). The peak of infection appeared around day 18 after intraperitoneal injection of the parasite in both models, and the highest parasite density reached about 55-60% on average and then fell back to 0 around 24-27 days (Fig. S1A & B, & Fig. S2). The dynamics of parasitemia and self-healing of the infection were consistent with those in our previous studies[16]. 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 incomparable between the two groups thereafter because most of the control mice died (Fig.1B). Importantly, the tumor-bearing mice treated with *Plasmodium* parasite showed better survival rate and longer overall survival compared with the control mice (Fig.1C) ($p < 0.001$). The tumor volume on day 15 after inoculation in *Plasmodium* treated group was significantly smaller than that in the control group (Fig.1E&F) ($p < 0.001$). The tumor weight on day 15 after inoculation in *Plasmodium* treated group was also significantly lower than that in the control group (Fig.1G&H) ($p < 0.001$). In addition, we found that there was almost no metastasis in the intraperitoneal of *Plasmodium* treated group, while there were many intestinal and abdominal metastases in the control group (Fig.1D) ($p < 0.001$).

***Plasmodium* infection remarkably suppressed HCC recurrence in the resection mouse model**

To characterize the effect of *Plasmodium* infection on HCC recurrence, the resection mouse model was established by intrahepatic implantation of tumor pieces and undergoing operation to completely remove tumors on day 21 after tumor implantation (Fig.2A). In the early stage of *Plasmodium* infection, there was no significant difference in weight between the two groups. After resection of tumor, weight loss and short-term weight recovery occurred in both groups. During the period of infection, the diet of mice in *Plasmodium* infection group was poor, thus the weight growth in *Plasmodium* infection group was slower

than that in the control group. After self-healing of infection, the weight in *Plasmodium* infection group continued to increase, while that of the control group decreased due to tumor development (Fig.2B). An effect of surgical resection followed by *Plasmodium* infection on weight loss was apparent while compared the infected group in resection model with the infected group in non-resection model (Fig.2B and Fig.1B). A phenomenon of hemozoin accumulation in the liver (dark color)[18] was found in *Plasmodium* infected group in either resection model (Fig.2D) or non-resection model (Fig.1E). Importantly, our results demonstrated that *Plasmodium* treated tumor-bearing mice survived much longer than the control mice (Fig.2C). The cumulative recurrence rate in *Plasmodium* infection group was much lower than that in control group on the 75th day (Fig.2D) ($p < 0.001$).

***Plasmodium* infection inhibited the metastasis of HCC potentially through suppression of EMT**

The EMT of HCC cells is closely associated with its ability of metastasis. E-cadherin is the key cadherin to prevent tumor metastasis and the most important biomarker of EMT, which is also crucial for HCC cells to maintain the normal cytoskeleton and the connection between homotypic cells [5]. Expectedly, the expression level of E-cadherin in *Plasmodium* treated group was significantly higher than that in control group (Fig.3, left & middle). The Snail superfamily of Zinc- finger transcription factors is a crucial transcription inhibitor for EMT, which can directly lead to inhibition of E-cadherin expression. As one of the biomolecules of E-boxes of the E-cadherin promoter, Snail could directly down-regulate the expression of E-cadherin and promote the occurrence of EMT [19]. Our results indicated that the expression of Snail showed a significant decrease in *Plasmodium*-treated group compared with control group (Fig.3, left & right).

***Plasmodium* infection suppressed the activation of PI3K/Akt/GSK-3 β signaling**

Previous studies have indicated that the PI3K/Akt/GSK-3 β signaling and the transcriptional repressor Snail were required for EMT of HCC [9, 11]. Activated downstream molecules of PI3K/Akt/GSK-3 β could mediate the stabilization of endogenous Snail. Therefore, we tested if *Plasmodium* infection had an influence on PI3K/Akt/GSK-3 β pathway in HCC cells. Western blot analysis showed that the phosphorylation of Akt (Fig. 4A&C) ($p < 0.05$) and GSK-3 β (Fig. 4A&D) ($p < 0.05$) in *Plasmodium*-treated group was downregulated, as compared with that in control group, even though the difference of PI3K lacked statistical significance between the two groups (Fig. 4B). These findings suggested that *Plasmodium* infection could inhibit the activation of PI3k/Akt/GSK-3 β signaling pathway, thus suppressed the accumulation of Snail and EMT programming.

***Plasmodium* infection downregulated the expression of CCR10**

Chemokines/chemokine receptors axis have been demonstrated to have the ability to activate PI3K/Akt/GSK-3 β /Snail signaling pathways in HCC cells[9, 11]. To specifically address the role of CCR10 mediated-PI3K/Akt/GSK-3 β /Snail signaling in *Plasmodium* infection, we examined the expression of CCR10 in tumor tissues derived from tumor-bearing mice. As expected, the mRNA expression level of CCR10 was significantly lower in *Plasmodium* treated group than that in control group (Fig.5B) ($p < 0.01$).

Western blot result showed that the expression level of CCR10 was also significantly lower in the infected group than that in the control group. (Fig.5A) ($p < 0.05$).

Discussion

Our present study indicates that *Plasmodium* infection significantly prolongs the survival time of tumor-bearing mice in both HCC models through inhibiting the tumor growth and abdominal metastasis. The underlying mechanisms of action might involve the inhibition of PI3K/Akt/GSK-3 β /Snail signaling by down-regulating the expression of CCR10, reduction of Snail accumulation, and up-regulation of E-cadherin.

Primary hepatocellular carcinoma ranks the fourth in the cause of cancer-related death worldwide. Although surgical resection can remove the visible tumor, it cannot cure liver cancer. Hepatoma cells can escape from the microenvironment and pass through blood vessels and lymphatics, which can lead to tumor recurrence and metastasis. This malignant behavior is the leading cause of postoperative death in patients with liver cancer [20]. Therefore, how to prevent metastasis of liver cancer cells and to reduce postoperative recurrence are the key element to improve the prognosis of patients with liver cancer. Our current study indicates that *Plasmodium* infection significantly reduces the postoperative recurrence and metastasis in the murine HCC resection model.

The epithelial–mesenchymal transition (EMT) has been implicated in carcinoma invasion and metastasis and is the initial step for epithelial cancer cells to escape from homologous cells [21]. Previous studies have suggested that down-regulation of E-cadherin is a prominent feature of EMT. It has been demonstrated that the transcriptional activity and protein expression of E-cadherin can be enhanced by preventing the binding of E-boxes of the E-cadherin promoter to transcriptional barriers (including Zinc-finger transcriptional repressors snail and slug, the repressor SIP-1/ZEB-2, DEF-1/ZEB-1, as well as the basic helix–loop–helix transcription factors twist and E12/E47), which prevent EMT [21, 22]. Our current study indicates that *Plasmodium* infection increased the expression of E-cadherin and downregulating Snail, which suggests that *Plasmodium* infection might prevent EMT in HCC.

PI3K activates and produces second messenger PIP3, which activates AKT through phosphorylation of AKT protein at Ser308 [23]. GSK-3 β is a downstream target of phosphorylated AKT, which promotes the degradation of Snail, while phosphorylation of GSK-3 β leads to its inactivation. Phosphorylated AKT can change GSK-3 β into phosphorylated GSK-3 β , which leads to the inactivation of GSK-3 β and the increase of Snail. Snail, as a transcription inhibitor of E-cadherin, is the downstream factor of GSK-3 β . GSK-3 β inhibits the expression of Snail and increases E-cadherin protein expression [24]. Our current study indicates that *Plasmodium* infection inhibits the phosphorylation of AKT, which inhibits the transition from GSK-3 β to phosphorylated GSK-3 β , and reduces the expression level of Snail protein. Finally, the inhibition effect of Snail on E-cadherin is removed and EMT might be prevented.

Chemokines are known as small (8 to 14 kDa) chemoattractant cytokines that selectively regulate the recruitment and trafficking of leukocyte subsets to the sites of inflammation. According to the spacing of

the first two cysteines in the N-terminus, chemokines are divided into several subfamilies—including C, CC, CXC, and CX3C chemokines. Chemokines exert its action through seven trans-membrane spanning G-protein-linked receptors (chemokine receptors) [25, 26]. Previous studies have suggested that chemokines and chemokine receptors are involved in inflammatory reactions and wound healing [27]. Recently, researches demonstrated that chemokines and their receptors play a critical role in tumor cell growth and metastasis in melanoma, lung cancer, gastric carcinoma, pancreatic cancer, colorectal carcinoma and hepatocellular carcinoma [28]. It has been reported that CCR10 activation stimulates the progression, invasion and migration of HCC, breast cancer cells and melanoma cells [11, 29, 30]. CCR10 expression and downstream PI3K/Akt pathway shows a role in HCC progression[11]. Our results showed that *Plasmodium* infection could downregulate the expression of CCR10. The inhibition of CCR10-mediated PI3K/Akt/GSK-3 β /Snail signaling might result in suppression of EMT programing in HCC.

Conclusions

In summary, our findings in current study might involve a possible new mechanism (Fig. 6) of *Plasmodium* infection against HCC in mice, provide a further evidence in prevention of reoccurrence and distant metastasis of HCC potentially by regulating EMT through infection with *Plasmodium* parasite. In addition, our previous studies have demonstrated that *Plasmodium* infection display antitumor effects through activating the innate and acquired antitumor immunity, remodeling tumor immunosuppressive microenvironment, and inhibiting angiogenesis with tumor [12-17]. Based on these studies, three clinical trials are ongoing in China (NCT02786589, NCT03474822 and NCT04165590). These findings may be particularly important for developing novel strategies for the treatment and prevention of metastasis and recurrence of hepatocellular carcinoma, especially for patients during the perioperative period.

Abbreviations

HCC: hepatocellular carcinoma; EMT: epithelial-mesenchymal transition; Tregs: regulatory T cells; MDSCs: myeloid-derived suppressor cells; CCR10: CC-chemokine receptors 10; Py: *Plasmodium yoelli* 17XNL; PI3K: phosphatidylinositol 3 kinase; GSK-3 β : glycogen synthase kinase-3 β ; RT-qPCR: quantitative real-time PCR

Declarations

Ethics approval and consent to participate

The animal experiment facilities were approved by the Guangdong Provincial Department of Science and Technology and complied with the guidelines of the Animal Care Committee, Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences. All efforts were made to minimize animal suffering.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its Additional files.

Competing interests

Authors declare no conflict of interest.

Funding

This study was supported by the Key Regional Project of Science and Technology Service Network Program of Chinese Academy of Sciences (NO. KFJ-STS-QYZX-042), Science and Technology Program of Guangzhou, China (NO. 201707010447), National Natural Science Foundation of China (NO. 81673003), Clinical Research Program of High Level University of Guangzhou Medical University 2017 and State Key Laboratory of Respiratory Diseases (NO. SKLRD-OP-201802) Applied Basic Research Projects of Yunnan Province, China [2019FE001-054].

Authors' contributions

Y.L. and X.C. conceived, planned and carried out this work together with Z.T., M.M., D.A., S.F., and Wenting Ding helped in the animal experiments. M.M. contributed to the molecular biology experiments. Linglin Dai, Xiaofen Li and Sting Zhao helped in reagent and material preparation. Xiaoping Chen, Li Qin and Xiaowen Zhang supervised this work. All authors discussed the results and contributed to the final manuscript. All authors read and approved the final manuscript.

Acknowledgment

We thank Dr. Zhenhui Li and Dr. Wei Guo for their assistance in animal experiments.

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Additional File Legends

Additional file 1

Fig S1. *Plasmodium* infection of red blood cell in the tumor resection model. (A) Microscopic view of parasitemia during infection, arrow indicates infected red blood cell (B) Parasitemia curve showing initiation, peak and decline timelines (n=13).

Additional file 2

Fig S2. Parasitemia curve showing initiation, peak and decline timelines in the tumor non-resection model (n=9).

Figures

Figure 1

Plasmodium infection significantly inhibited HCC progression and metastasis in the non-resection mouse model. **A** Mice successfully implanted with Hepa1-6 cells and confirmed by fluorescence through detection of IVIS Spectrum. **B** Body weight curve of mice in Hep and Hep + Py groups (n=26). **C** Survival curve of mice in Hep and Hep + Py groups (n=26). **D** Example of metastatic nodules in the abdominal cavity. Tumor metastasis in Hep and Hep + Py groups was measured 15 days after tumor cell inoculation (n=26). **E** Example of HCC nodules in the liver. **F** The tumor volume in Hep and Hep + Py groups measured 15 days after tumor cell inoculation (n=26). **G** Plot of tumor weight/body weight ratio of Hep and Hep + Py measured 15 days after tumor cell inoculation (n=26). **H** Plot of tumor weight/liver weight ratio of Hep and Hep + Py measured 15 days after tumor cell inoculation (n=26).

Figure 2

Plasmodium infection suppressed HCC progression and recurrence in the resection mouse model. 5 x 10⁵ Hepa1-6 cells were s.c. injected into the right flank of 2 C57BL/6 mice. Fourteen days post inoculation, subcutaneous tumors were surgically harvested and divided into small pieces. The resection mouse model was established by intrahepatic implantation of the isolated tumor pieces; 21 days after tumor implantation, tumors were carefully resected. Mice were followed for an additional 3 days to allow for recovery, then half of the mice were randomly infected with *P. yoelii* 17XNL (Py) or injected with nothing as control. **B** Body weight curve of mice in Hep and Hep + Py groups (n=18). **C** Survival curve of mice in Hep and Hep + Py groups (n=18). **D** Example of metastatic nodules in the abdominal cavity and comparison of tumor recurrence or metastasis between the two groups. The cumulative recurrence or metastasis rate in Plasmodium infection group was much lower than that in control group on the 75th day (n=18).

Figure 3

Plasmodium infection downregulated the expression level of Snail and upregulated the expression level of E-cadherin. Western blot analysis of the levels of E-cadherin and Snail in the tumor tissues of the treated and control groups. The experiments were repeated one time and the results were similar.

Figure 4

Plasmodium infection suppressed the activation of PI3K/AKT/GSK-3β signaling. (A) Western blot analysis of phosphorylated (p-) PI3K, AKT, p-AKT, GSK-3β and p-GSK-3β in Plasmodium treated group and in control group. (B) Comparison of protein levels of p-PI3K between two groups. (C) Comparison of p-

AKT between two groups. (D) Comparison of p-GSK-3 β between two groups. The experiments were repeated once and the results were similar.

Figure 5

Plasmodium infection downregulated the expression of CCR10. (A) Western blot result of CCR10. (B) The mRNA expression level of CCR10. The experiments were repeated once and the results were similar.

Figure 6

Possible mechanism of Plasmodium infection against recurrence and metastasis of HCC in mice. Plasmodium infection induces: (1) downregulation of CCR10 and p-AKT, but not PI3K; (2) downregulation of p-GSK-3β, but not GSK-3β; (3) downregulation of Snail, but not p-Snail; (4) upregulation of E-cadherin. Importantly, downregulation of Snail and upregulation of E-cadherin might contribute to the suppression of EMT in murine HCC resection and non-resection models.

Supplementary Files

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