Multitargeting Strategy Using Tetrathiomolybdate and Lenvatinib: Maximizing Anti-Angiogenesis Activity in a Preclinical Liver Cancer Model

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Research Article

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Abstract

Purpose

To investigate the suppressing tumor-promoting effects via multi-anti-angiogenesis activity of the copper chelator (Ammonium Tetrathiomolybdate, TM) combined with lenvatinib for hepatocellular carcinoma.

Material and methods

Fifty-five C57 mice were injected subcutaneously with Hepa1-6 hepatoma cell suspensions into the right posterior thigh. Seven days later, all subcutaneous tumors were formed and the mice were randomly distributed into 5 groups: TM Group (G1), Lenvatinib Group (G2), TM+Lenvatinib Group (G3), Control Group (G4), and Copper (II) Gluconate Group (G5). And copper concentrations in serum and tumors were measured at the predetermined times. After fourteen days of treatments, tumor weight and volumes were analyzed, histology was observed, and the expressions of VEGF and microvessel density (MVD) in tumor tissues were measured by immunohistochemistry (IHC).

Results

Average concentration of copper in serum was 405.14 ug/L, 480.44 ug/L, and 679.80 ug/L in normal mice, in mice on 7 days after implantation, and in the control group, respectively. Similarly, intratumoral copper concentrations were greater in G4 mice (1511.90 ug/L) than mice on 7 days after implantation (852.80 ug/L) ($p < 0.05$). And the serum concentration of copper was 363.65 ug/L, 508.83 ug/L, 370.52 ug/L, 822.12 ug/L in G1, G2, G3, and G5 [G5 vs other Groups, all $p < 0.05$; (G1, G2, and G3) vs G4, $p < 0.05$; G1 vs G2, $p = 0.013$; G2 vs G3, $p = 0.018$; G1 vs G3, $p = 0.903$] while intratumoral copper concentrations was 674.31 ug/L, 988.91 ug/L, 550.52 ug/L, and 3004.95 ug/L in G1, G2, G3, and G5 And the average tumor weight was 0.55 g, 0.44 g, 0.08 g, 1.37 g, 3.11 g in the mice of G1, G2, G3, G4, and G5, respectively. [G5 vs other Groups, all $p < 0.05$; (G1, G2, and G3) vs G4, $p < 0.05$; G1 vs G3, $p < 0.05$; G2 vs G3, $p < 0.05$; G1 vs G2, $p > 0.05$]. Furthermore, tumors were collected for HE staining and IHC examination. The expression levels of VEGF in G1, G2, and G3 were 43.75, 32.48, and 15, and all of them were significantly lower than those in G4 (64.28) and G5 (89.03) (G4 vs G5, $p < 0.05$). Similarly, the trend of MVD was just like that of VEGF in the five groups whereas no significant difference occurred in G1 and G2.

Conclusion

The study shows that there is a significant positive correlation between tumor load and copper. Administration of copper can promote tumor progression, and copper chelating could suppress tumor growth. The combination of TM with lenvatinib can reduce tumor angiogenesis and improved the effect

of antitumor treatment. These findings offer basic data support and theoretical foundation for the clinical application of the combination therapy.

1. Introduction

Hepatocellular carcinoma (HCC) is the second most lethal tumor and ranks sixth in terms of incident cases with a 5-year survival of 18% in the world [1, 2]. It is a kind of hypervascular tumors fed by multiple malformed arteries [3] and as one of the hallmarks in cancer, angiogenesis is closely related to the malignant behaviors [4, 5]. Therefore, there have been numerous efforts in targeting important cellular proteins in cancer angiogenesis with some encouraging results [6, 7].

Lenvatinib, as a new effective option for frontline therapy of advanced HCC, showed antitumoral activity and improved the median overall survival (OS) from 12.3 months with sorafenib, the first systemic drug approved by the Food and Drug Administration (FDA) for the treatment of HCC [8], to 13.6 months [6]. However, studies have shown that targeting one or two proteins in the complex cancer cascade may not be sufficient in controlling and inhibiting cancer growth [7, 9]. In advanced HCC, the improvements in progression-free survival and overall survival achieved with lenvatinib are considered to be modest [10]. Therefore, there is an urgent need to target more potential facets of cancer angiogenesis.

Copper is an essential trace element that plays a central role in the biochemistry of every living organism [11, 12]. The role of excess copper in both the etiology and growth of different kinds of tumors has been extensively studied. It has been reported that both the serum and tumor copper levels are elevated in a plethora of malignancies including lymphoma, reticulum cell sarcoma, bronchogenic and laryngeal squamous cell carcinomas, cervical, breast, stomach, lung cancers, etc.[11, 13–15] A recent study demonstrated that higher serum copper may be associated with worse survival in HCC patients [16]. Additionally, elevated copper levels can facilitate angiogenesis, cancer growth, and metastasis via activating numerous pro-angiogenic factors, such as VEGF, basic fibroblast growth factor (bFGF), tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1, IL-6, IL-8, fibronectin, *et al*. [17–20] Therefore, the hypothesis that copper chelators can be employed as an anti-angiogenic agent is feasible [21–25]. Interestingly, copper chelators have been shown to inhibit tumor angiogenesis and growth in animal models and clinical trials [25–28]. However, it was confirmed that copper chelators showed only limited efficacy on tumor progression and angiogenesis in advanced cancer [27, 29]. Therefore, it is worth exploring whether the combination of the two drugs can be more sufficient in controlling HCC angiogenesis and tumor growth. Hence, the present study aimed to investigate the maximizing anti-angiogenesis activity of copper chelators combined with lenvatinib for HCC.

2. Materials And Methods

2.1 Materials

Hepa1-6 hepatoma cells was purchased from ATCC. Anti-VEGF antibody (rabbit polyclonal IgG, 1:200 dilution, Cat. no.BM3995) was obtained from Boster Biological Technology (Wuhan, China). CD34 (rabbit polyclonal IgG, 1:250 dilution, ab-81289) was purchased from Abcom Inc. Ammonium tetrathiomolybdate (TM, 99.95%) (Cat. no. G1915025) was purchased from Aladdin Biochemical Technology Co., Ltd (Shanghai, China). Copper (II) gluconate (98%) (Cat. no. C10070830) was purchased from Mackling Biochemical Co., Ltd (Shanghai, China). A total of 60 female 5-week-old C57BL/6j mice were purchased from Guangdong Medical Laboratory Animal Center (Guangzhou, China). They were housed under controlled temperature conditions and relative humidity, with 10–15 air changes per hour and light illumination for 12 hr. a day. The animals were allowed free access to food and water throughout the acclimation and experiment protocols. All animal procedures were performed according to approved protocols (NO: SYSU-IACUC-2020-000208) and in accordance with the recommendations for the proper care and use of laboratory animals.

2.2 Animal treatments

In total, 60 C57BL/6j mice were used for our study. To create the xenograft model, Hepa1-6 tumor cells (1×10^6 cells) were injected into the flank of 55 C57mice. When the mean tumor volume reached approximately 100 ± 50 mm^3 7 days after injection, tumor-bearing mice were randomized and divided into five experimental groups. Group 1 (G1) was given TM at a dose of 1.25 mg/day once daily by gavage [30] (n = 10). Group 2 (G2) was given a gavage of lenvatinib (30 mg/kg, body weight) [31] (n = 10). Group 3 (G3) was given a gavage of lenvatinib combined with TM (n = 10). Group 4 (G4) was the control group without any treatment (n = 10). Aqueous solutions of Copper(II) gluconate at the dosage of 0.15mg/kg [32] was administered to Group 5 (G5)(n = 10). Lenvatinib and TM were administered for 14 days. Tumor volumes (mm^3) were calculated every three days using a vernier caliper in 2 dimensions as previously described [31] and was calculated using the formula: length (mm) × width $(mm)^2$ × 1/2.

2.3 Pathological investigations

Five normal mice and five mice on 7 days after implantation were anesthetized by intraperitoneal injection of 50 mg/kg of pentobarbital sodium for conventional blood collection. After 14 days of treatment, all the animals were also anesthetized for blood collection and the tumors were excised and weighed. Then serum copper concentrations were quantified using inductively coupled plasma mass spectrometry (ICP-MS) (Agilent 7700x ICP-MS spectrometer, Agilent Technologies, Germany) [16].

Each tumor was cut into two sections, in which one was fixed in 4% paraformaldehyde for histologic examination and immunohistochemistry (IHC), whereas the other was used to quantify intratumoral copper concentration.

Intratumoral copper concentrations were determined in the tissue specimens using slight modifications of previously described methodology [33]. In briefly, fresh tumor tissues were packed into frozen pipes and stored at −20°C for copper analysis. Approximately 0.1 g samples were digested in 3 mL of concentrated nitric acid and 1 mL 30% w/v hydrogen peroxide in a microwave digestion system. Digested samples

were transferred to sample tubes and diluted to 3 mL with ultrapure water. The concentrations of copper were determined by an Agilent 7500 inductively coupled plasma-mass spectrometer (ICP-MS; Agilent 7700x ICP-MS spectrometer, Agilent Technologies, Germany).

The other tumor samples were embedded with paraffin, sectioned into 3 μm sections, dewaxed, rehydrated using xylene and successive ethanol baths, immersed in 10 mM Tris and 0.5 M EDTA at pH 9.0, and then microwaved twice for 5 min each. Subsequently, the sections were incubated with 3% H_2O_2 for 10 min to block endogenous peroxidase activity. The sections were then incubated overnight at 4°C with specific primary antibody (dilutions: VEGF2, 1:200; CD34, 1:200) in a humidified atmosphere. The sections were scanned using a Leica Aperio AT2 (Leica Biosystems, Germany) scanner, and the images were analyzed using ImageJ software.

The expression of VEGF and microvessel density (MVD) labeled by CD34 was independently reviewed by two experienced pathologists blinded to every slide. The expression of VEGF was determined by counting stained epithelial cells in five random sights of every slide (400 magnification) as previously described [34]. Intratumoral MVD was assessed with CD34 staining using the vascular hotspot technique as described by Weidner, *et al.* [34, 35] Sections were scanned at low power to determine areas of highest vascular density and stained microvessels were counted in five separate random fields at high power (400 magnification) within this region. The mean vessel count from the five fields was used. A single countable microvessel was defined as any endothelial cell or cell cluster showing positive CD34 staining without the necessity of a vessel lumen.

2.4. Statistical Analysis

The results were calculated by averaging data from multiple measurements and were presented as mean ± standard deviations (SD). One-way analysis of variance (ANOVA) complemented using SPSS software package (Version 25.0) for differences between means was used to detect any significant difference ($P < 0.05$) among different groups in this study. Person's rank correlation coefficients were used to estimate the correlations of copper concentration and tumor neovascularization.

3. Results

3.1 Antitumor activity of TM and/or lenvatinib in Hepa1-6 xenograft models

To examine the antitumor activity of lenvatinib and/or TM, we established a xenograft tumor derived from Hepa1-6 cells in mice. The mice were treated with TM (1.25 mg/day) and/or lenvatinib (30 mg/kg) or copper gluconate (0.15mg/kg) for 2 weeks beginning at 7 days after tumor cell inoculation, and the tumor volumes were measured every three days. The tumor weights were 0.55±0.49 g, 0.44±0.21 g, 0.08±0.08 g, 1.37±0.38 g, and 3.11±0.78 g and the volumes were 976.77 ± 722.51mm^3, 818.81 ± 458.59

mm^3, 203.84±84.1 mm^3, 1816.16±560.43 mm^3, and 4394.98±1058.63 mm^3 in the mice of G1, G2, G3, G4, and G5, respectively. As shown in Fig. 1, the Hepa1-6 xenograft tumor volumes and weight were significantly decreased in the TM-, lenvatinib-, and TM+lenvatinib-treated mice compared to the untreated mice (control group) and copper-treated group with a significant difference ($p < 0.05$). Compared with the control group (G4) and other treatment groups (G1, G2 and G3), mice in the copper-treated group (G5) had the largest tumor volume and weight with statistical significance (all $p < 0.05$). Furthermore, TM treatment led to a positive trend by enhancing the antitumor effects of lenvatinib in terms of tumor volume and weight (TM single treatment and lenvatinib single treatment vs. combined treatment, all $p < 0.05$). However, no statistical differences were observed between lenvatinib single treatment and TM single treatment in regards to tumor volume and weight ($p > 0.05$) though tumor volume and weight of the lenvatinib-treated group were smaller than that of the TM-treated group. These results suggest that antitumor effects of combination treatment with TM and lenvatinib have better anticancer activity. No significant toxicity was observed during the treatment period.

3.2 Serum and Intratumoral Copper Concentrations in mice with different interventions

Blood samples for assessment of copper were obtained in normal mice, xenograft mice on 7 days after implantation of Hepa1-6 cells, and in mice at 14 days after treatments and tumor samples were obtained in xenograft mice on 7 days after implantation, and in mice at 14 days after treatments using LC–MS/MS methods.

The average serum concentration of copper was 405.14 ug/L (SD=52.73) in normal mice (day 0) (Fig. 2). The concentration was 480.44 ± 40.08 ug/L and 852.80 ± 44.63 ug/L at 7 days after implantation and 679.80 ± 47.65 ug/L and 1511.90 ± 452.51 ug/L in serum and tumor in mice of the control group. Therefore, the study has demonstrated a significant elevation of the mean total serum copper concentrations as the tumor grew in mice without drug intervention. In Group5, the concentration of copper (822.12 ± 110.82 ug/L in serum and 3004.95 ± 178.10 ug/L in the tumor) reached their highest values after a 14-day administration of copper (II) gluconate compared to other groups (Fig. 2) (all $p < 0.05$). The mean total serum copper concentrations [(G1 363.65 ± 117.29 ug/L, G2 508.83 ± 52.39 ug/L, G3 370.52 ± 224.17 ug/L) vs (G4 679.80 ± 47.65 ug/L, G5 822.12 ± 110.82 ug/L), all $p < 0.05$] and intratumoral copper concentrations [(G1 674.31 ± 157.75 ug/L, G2 988.91 ± 111.59 ug/L, G3 550.52 ± 375.79 ug/L) vs (G4 1511.90 ± 452.51 ug/L, G5 3004.95 ± 178.10 ug/L), all $p < 0.05$] were found significantly decreased in G1, G2, and G3 compared with G4 and G5 with statistical differences. The significant difference in the mean serum copper concentrations between TM-treated, combined groups (G1, G3) and lenvatinib-treated group (G2) was observed (G1 vs G2, $p = 0.013$; G2 vs G3, $p = 0.018$) while no statistical significance between G1 and G3 ($p = 0.903$) occurred. Similarly, the significant difference in the mean intratumoral copper concentrations between G2 and G3 was observed (G2 vs G3, $p=0.017$) while there was no statistical significance between G1 and G2 ($p = 0.078$) and G1 and G3 ($p = 0.475$).

3.3 Pathomorphological changes of tumor tissues

After staining by hematoxylin-eosin (HE), tumor tissue sections showed deeper purple color and tumor cells were concentrated with confused and disordered distribution (Fig. 3). The section showed light purple color and scattered tumor cells in TM- and/or lenvatinib-treated groups. Furthermore, a large eosinophilic patch in the visible area was the tumor necrosis area (Fig. 3B). However, several small vessels and red blood cells were seen in copper-treated group and higher disorder degree of tumor cell distribution, and intensive degree of tumor cells were shown when compared to while the TM- and/or lenvatinib-treated groups and control group (Fig. 3).

3.4 IHC analysis of tumor vasculature (VEGF and MVD) as biomarkers associated with the antitumor activity of different treatments

We examined VEGF which promotes neovascularization and MVD tagged by CD34 as the biomarker of endothelial cells using tumor specimens to determine the pro-angiogenesis effect of copper by IHC in tumor xenograft models.

After the 14-day treatment period, the tumors were excised and examined histologically. As is shown in Fig. 4 **and** Fig. 5, VEGF protein in tumor tissue was stained to brown-yellow color and the positive expression area of MVD, which is mainly expressed on the vascular endothelial cell membrane, was showed deep brown color. The expression of VEGF and MVD was (43.75 ±17.83, 32.48 ± 7.73, 15.00 ± 6.05, 64.28 ± 11.45, 89.03 ± 20.39) and (44.25 ± 9.65, 41.2 ± 8.61, 20.67 ± 7.11, 85.52 ±10.38, 109.71 ± 12.16) in tumor specimens of G1, G2, G3, G4, G5, respectively. As shown in Fig. 4F and Fig. 5F, the immunohistochemical images indicated a descending expression level of both VEGF and MVD in TM and/or lenvatinib (G1, G2, G3) as compared to control (G4) and copper-treated group (G5) with statistical differences [(G1, G2, and G3) vs (G4, G5), all $p < 0.05$]. The expression of VEGF and MVD in G5 were higher than control group, which demonstrated that the effects of copper on promoting VEGF expression and subsequent new vessels development (G5 vs other Groups, all $p < 0.05$). Furthermore, tumors in G3 had the lowest median VEGF and MVD when compared with G1 and G2 with significant differences [G1 vs (G2, G3), $p < 0.05$]and there were also statistical differences between G1 and G2 in terms of VEGF while no significant difference occurred in G1 and G2 in terms of MVD. These results suggested copper chelators combined with lenvatinib had a better effect on anti-angiogenesis activity.

3.5 Correlation between tumor angiogenesis and copper in xenograft tumors

VEGF and MVD are commonly utilized to assess tumor angiogenic activity and they are consistently associated with correlational coefficient 0.907, which was positive. A significant statistical correlation (VEGF and serum copper, R = 0.876; MVD and serum copper, R= 0.764; VEGF and intratumoral copper, R = 0.775; MVD and intratumoral copper, R= 0.696) was found between copper concentration and the expression of VEGF and MVD (all $p < 0.001$), and the correlation between their expression was confirmed

by the Pearson's correlation analysis of continuous random variables. The correlation between copper and tumor volumes was also positive (weight and serum copper, R= 0.895; weight and intratumoral copper, R= 0.739; volume and serum copper, R= 0.873; volume and intratumoral copper, R= 0.66). Furthermore, the expression of VEGF and MVD were correlated with tumor volumes (R= 0.834 and 0.727, all $p < 0.001$).

4. Discussion

Liver cancer is a highly aggressive malignancy with poor prognosis. At present, combined treatment of liver cancer has been the prevalent trend, such as systemic therapies combined with TACE (transcatheter arterial chemoembolization) or ablation and achieved good results [36–38]. Systemic therapies have gradually become the basic therapy and standard treatments in advanced hepatocellular carcinoma (HCC) because tumor angiogenesis is a crucial factor closely associated with tumor growth, progression, and metastasis[39]. In this regard, numerous efforts have been made to explore the possibilities of curing cancer by targeting angiogenesis.

Conventional approaches comprise the administration of anti-angiogenic drugs that target and block the activity of proangiogenic factors. Lenvatinib, an angiogenesis inhibitor targeting tyrosine kinase, is a first-line treatment for advanced HCC with proven survival benefit [6, 31]. In our study, lenvatinib can effectively reduce tumor volume and weight via inhibiting tumor angiogenesis measured with VEGF and MVD when compared with the control group. The results are consistent with those reported by Yuji Yamamoto, *et al*. [31] However, the partial response of lenvatinib was only 18% and the median overall survival remains ~1 year [6]. As is known to us all, hepatocarcinogenesis is a multi-factorial process with various signaling pathways implicated in tumor growth and metastasis. Vascular endothelial growth factor (VEGF) signaling, epidermal growth factor (EGF) signaling, Ras MAPK signaling, PI3K/PTEN/Akt/Mtor pathway, HGF/c-MET pathway, insulin-like growth factor receptor (IGFR) signaling, Wnt/b-Catenin pathway, apoptotic signaling pathways, hedgehog signaling pathway, Jak/STAT pathway, etc. are some of the major pathways implicated in carcinogenesis [7, 40–42]. And it was reported that new treatment protocol with alternating sorafenib and lenvatinib for refractory thyroid cancer was more effective than individual treatment with sorafenib or lenvatinib [43]. Hence, targeting only one of these multiple pathways with singly-targeted drugs is highly unlikely to be effective and a combination of treatments is the way to go [7, 9].

Studies have demonstrated the association between copper and tumor angiogenesis[17]. In the study, we detected the copper concentration of serum and tumor at different points after implantation using ICP-MS. The results demonstrated that serum copper was significantly higher than those of normal mice after implantation and the copper concentration of serum and tumor increased with increased tumor size. This suggests that copper may promote the rapid growth of tumors. To further verify the relationship between copper and tumor growth, Copper(II) Gluconate and the copper chelator (Tetrathiomolybdate, TM) were administered to explore the changes of tumor volumes and weights in experimental animals. TM, which

was developed for the treatment of Wilson's disease, depleting copper levels in tumors was reported to treat tumors as an anti-cancer and anti-angiogenic agent [44]. And the results proved that copper can promote liver tumor growth, whereas tumor growth slowed down significantly after the administration of TM. And correlation analysis was carried out to further confirm the conclusion. The results showed that copper concentration was positively correlated with tumor loads. In order to study the correlation between copper and tumor angiogenesis, VEGF and MVD were selected as the biomarkers of neovascularization. The results reveal that the expressions of VEGF and MVD were significantly increased after the administration of copper (89.03, 109.71), and decreased after the treatment of TM (43.75, 44.25) when compared to the control group (64.28, 85.52) with statistical significance. And there was a significant positive correlation between copper concentration and tumor neovascularization index via correlation analysis. Studies were reported that TM was regarded as antiangiogenic agents because of dual mechanisms of action. The first involves reducing copper stores and availability, rendering the copper unavailable for cellular uptake and therefore preventing it from participating in angiogenesis [30]. Secondly, TM has major anti-inflammatory properties by inhibiting copper-dependent cytokines involved in inflammation and its anti-inflammatory effect may be involved in TM's anticancer effect because cancers attract inflammatory cells that provide a plethora of additional proangiogenic agents [29]. Although TM showed excellent efficacy on many mouse cancer models, TM showed only limited efficacy in advanced cancer [29]. The major reason maybe be that TM inhibits angiogenesis by inhibiting key copper-dependent angiogenic promoters. It appears that micro clusters of cancer cells have only several angiogenic promoters available, and it, or they, is copper-dependent and therefore inhibited by TM. In contrast, bulky cancers attract inflammatory cells which bring in many angiogenic promoters, some of which are not copper-dependent, and allow cancer to grow irrespective of TM therapy. As their efficacy is still a matter of debate, novel strategies have been focusing on combining anti-angiogenic agents with copper chelators. The dramatic effects of TM on mouse cancer models and the main purpose of the combination of lenvatinib and TM is that TM can be used for smaller liver cancer lesions because the tumor was small, micro, clusters of cancer cells while lenvatinib can act directly on advanced liver cancer. In our study, the results showed that the combination of TM and lenvatinib could significantly slow down the tumor growth rate, reduce the tumor load when compared to the TM single treatment and lenvatinib single treatment. To further confirm the therapeutic effect of the combination regimen and explore the mechanisms by which the combining therapy inhibited tumor growth and the effect on tumor angiogenesis, VEGF and MVD in tumor tissue with different interventions were investigated by immunohistochemistry (IHC) assay. The results showed that the combined therapy could not only reduce the concentration of copper in the tumor, but also reduce tumor angiogenesis. Preliminary results of the study also proved that single-agent antiangiogenic therapy is poorly active in advanced tumors, because antiangiogenic agents aiming at single targets can be neutralized by up-regulation of other proangiogenic factors [45, 46]. And combined approaches addressing at least two angiogenic targets should be more effective. TM enhances the antineoplastic activity of lenvatinib, this suggests a possible avenue to evaluate TM clinically.

5. Conclusion

Our preclinical study suggests that administration of copper can promote tumor progression, and copper chelating could suppress tumor growth. Copper accumulation could be involved in the angiogenesis of liver cancer in mice while the administration of copper chelating could suppress decrease tumor angiogenesis. The combination of a TM with lenvatinib reduced tumor angiogenesis and had a good anticancer effect, far better than any involved monotherapy. These findings offer another basic data support and theoretical foundation for the clinical application of the combination therapy.

Declarations

Availability of data and materials

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

Competing interests

There are no conflicts of interest to declare.

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Contributions

LN and HYH responsible for the conception and design. YJY and HYH gave administrative support. LN and WY did animal procedures, the collection and assembly of data. LN and HYH carried out the data analysis and interpretation. All authors contributed in the manuscript writing. The authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal procedures were performed according to approved protocols (NO: SYSU-IACUC-2020-000208) and in accordance with the recommendations for the proper care and use of laboratory animals.

Consent for publication

Not applicable.

References

1. Villanueva A. Hepatocellular Carcinoma. N Engl J Med. 2019;380(15):1450–1462.
2. Jemal A, Ward EM, Johnson CJ, et al. Annual Report to the Nation on the Status of Cancer, 1975-2014, Featuring Survival. J Natl Cancer Inst. 2017;109(9).
3. Yang ZF, Poon RTP. Vascular changes in hepatocellular carcinoma. Anat Rec (Hoboken). 2008;291(6):721–734.
4. Fu Y, Liu S, Zeng S, et al. From bench to bed: the tumor immune microenvironment and current immunotherapeutic strategies for hepatocellular carcinoma. J Exp Clin Cancer Res. 2019;38(1):396–396.
5. Liu K, Min X-L, Peng J, et al. The Changes of HIF-1α and VEGF Expression After TACE in Patients With Hepatocellular Carcinoma. J Clin Med Res. 2016;8(4):297–302.
6. Kudo M, Finn RS, Qin S, et al. Lenvatinib versus sorafenib in first-line treatment of patients with unresectable hepatocellular carcinoma: a randomised phase 3 non-inferiority trial. Lancet. 2018;391(10126):1163–1173.
7. Teleanu RI, Chircov C, Grumezescu AM, et al. Tumor Angiogenesis and Anti-Angiogenic Strategies for Cancer Treatment. J Clin Med. 2019;9(1):E84.
8. Llovet JM, Ricci S, Mazzaferro V, et al. Sorafenib in advanced hepatocellular carcinoma. N Engl J Med. 2008;359(4):378–390.
9. Wang Z, Dabrosin C, Yin X, et al. Broad targeting of angiogenesis for cancer prevention and therapy. Semin Cancer Biol. 2015;35 Suppl(Suppl):S224-S243.
10. Llovet JM, Montal R, Sia D, et al. Molecular therapies and precision medicine for hepatocellular carcinoma. Nat Rev Clin Oncol. 2018;15(10):599–616.
11. Gupte A, Mumper RJ. Elevated copper and oxidative stress in cancer cells as a target for cancer treatment. Cancer Treat Rev. 2009;35(1):32–46.
12. Grubman A, White AR. Copper as a key regulator of cell signalling pathways. Expert Rev Mol Med. 2014;16:e11.
13. Blockhuys S, Wittung-Stafshede P. Copper chaperone Atox1 plays role in breast cancer cell migration. Biochem Biophys Res Commun. 2017;483(1):301–304.
14. Jana A, Das A, Krett NL, et al. Nuclear translocation of Atox1 potentiates activin A-induced cell migration and colony formation in colon cancer. PLoS One. 2020;15(1):e0227916-e0227916.
15. Kim Y-J, Bond GJ, Tsang T, et al. Copper chaperone ATOX1 is required for MAPK signaling and growth in BRAF mutation-positive melanoma. Metallomics: integrated biometal science. 2019;11(8):1430–1440.
16. Fang AP, Chen PY, Wang XY, et al. Serum copper and zinc levels at diagnosis and hepatocellular carcinoma survival in the Guangdong Liver Cancer Cohort. Int J Cancer. 2019;144(11):2823–2832.
17. Denoyer D, Masaldan S, La Fontaine S, et al. Targeting copper in cancer therapy: 'Copper That Cancer'. Metallomics. 2015;7(11):1459–1476.

18. Rigiracciolo DC, Scarpelli A, Lappano R, et al. Copper activates HIF-1α/GPER/VEGF signalling in cancer cells. Oncotarget. 2015;6(33):34158–34177.
19. Finney L, Vogt S, Fukai T, et al. Copper and angiogenesis: unravelling a relationship key to cancer progression. Clin Exp Pharmacol Physiol. 2009;36(1):88–94.
20. Carmeliet P, Jain RK. Molecular mechanisms and clinical applications of angiogenesis. Nature. 2011;473(7347):298–307.
21. Khan MK, Miller MW, Taylor J, et al. Radiotherapy and antiangiogenic TM in lung cancer. Neoplasia. 2002;4(2):164–170.
22. Moriguchi M, Nakajima T, Kimura H, et al. The copper chelator trientine has an antiangiogenic effect against hepatocellular carcinoma, possibly through inhibition of interleukin-8 production. Int J Cancer. 2002;102(5):445–452.
23. Yoshida D, Ikeda Y, Nakazawa S. Suppression of tumor growth in experimental 9L gliosarcoma model by copper depletion. Neurol Med Chir (Tokyo). 1995;35(3):133–135.
24. Brem SS, Zagzag D, Tsanaclis AM, et al. Inhibition of angiogenesis and tumor growth in the brain. Suppression of endothelial cell turnover by penicillamine and the depletion of copper, an angiogenic cofactor. Am J Pathol. 1990;137(5):1121–1142.
25. Pan Q, Kleer CG, van Golen KL, et al. Copper deficiency induced by tetrathiomolybdate suppresses tumor growth and angiogenesis. Cancer Res. 2002;62(17):4854–4859.
26. Khan G, Merajver S. Copper chelation in cancer therapy using tetrathiomolybdate: an evolving paradigm. Expert Opin Investig Drugs. 2009;18(4):541–548.
27. Jain S, Cohen J, Ward MM, et al. Tetrathiomolybdate-associated copper depletion decreases circulating endothelial progenitor cells in women with breast cancer at high risk of relapse. Ann Oncol. 2013;24(6):1491–1498.
28. Chan N, Willis A, Kornhauser N, et al. Influencing the Tumor Microenvironment: A Phase II Study of Copper Depletion Using Tetrathiomolybdate in Patients with Breast Cancer at High Risk for Recurrence and in Preclinical Models of Lung Metastases. Clinical cancer research: an official journal of the American Association for Cancer Research. 2017;23(3):666–676.
29. Brewer GJ. The promise of copper lowering therapy with tetrathiomolybdate in the cure of cancer and in the treatment of inflammatory disease. J Trace Elem Med Biol. 2014;28(4):372–378.
30. Brewer GJ, Merajver SD. Cancer therapy with tetrathiomolybdate: antiangiogenesis by lowering body copper–a review. Integr Cancer Ther. 2002;1(4):327–337.
31. Yamamoto Y, Matsui J, Matsushima T, et al. Lenvatinib, an angiogenesis inhibitor targeting VEGFR/FGFR, shows broad antitumor activity in human tumor xenograft models associated with microvessel density and pericyte coverage. Vasc Cell. 2014;6:18.
32. Zhou B, Guo L, Zhang B, et al. Disulfiram combined with copper induces immunosuppression via PD-L1 stabilization in hepatocellular carcinoma. Am J Cancer Res. 2019;9(11):2442–2455.

33. Cedeño Y, López-Alonso M, Miranda M. Hepatic concentrations of copper and other metals in dogs with and without chronic hepatitis. J Small Anim Pract. 2016;57(12):703–709.
34. Li N, Chen B, Lin R, et al. The earlier, the better: the effects of different administration timepoints of sorafenib in suppressing the carcinogenesis of VEGF in rats. Cancer Chemother Pharmacol. 2018;81(1):207–216.
35. Weidner N, Semple JP, Welch WR, et al. Tumor angiogenesis and metastasis–correlation in invasive breast carcinoma. N Engl J Med. 1991;324(1):1–8.
36. Peng Z, Chen S, Xiao H, et al. Microvascular Invasion as a Predictor of Response to Treatment with Sorafenib and Transarterial Chemoembolization for Recurrent Intermediate-Stage Hepatocellular Carcinoma. Radiology. 2019;292(1):237–247.
37. Zhang T, Huang W, Dong H, et al. Trans-catheter arterial chemoembolization plus Sorafenib, an unsuccessful therapy in the treatment of hepatocellular carcinoma?: A systematic review and meta-analysis. Medicine (Baltimore). 2020;99(29):e20962.
38. Couri T, Pillai A. Goals and targets for personalized therapy for HCC. Hepatol Int. 2019;13(2):125–137.
39. Zhu XD, Tang ZY, Sun HC. Targeting angiogenesis for liver cancer: Past, present, and future. Genes Dis. 2020;7(3):328–335.
40. Branda M, Wands JR. Signal transduction cascades and hepatitis B and C related hepatocellular carcinoma. Hepatology. 2006;43(5):891–902.
41. Kudo M. Signaling pathway/molecular targets and new targeted agents under development in hepatocellular carcinoma. World J Gastroenterol. 2012;18(42):6005–6017.
42. Llovet JM, Villanueva A, Lachenmayer A, et al. Advances in targeted therapies for hepatocellular carcinoma in the genomic era. Nat Rev Clin Oncol. 2015;12(7):408–424.
43. Kim SY, Kim SM, Chang HJ, et al. SoLAT (Sorafenib Lenvatinib alternating treatment): a new treatment protocol with alternating Sorafenib and Lenvatinib for refractory thyroid Cancer. BMC Cancer. 2018;18(1):956.
44. Pan Q, Bao LW, Merajver SD. Tetrathiomolybdate inhibits angiogenesis and metastasis through suppression of the NFkappaB signaling cascade. Mol Cancer Res. 2003;1(10):701–706.
45. Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell. 1996;86(3):353–364.
46. Gasparini G, Longo R, Fanelli M, et al. Combination of antiangiogenic therapy with other anticancer therapies: results, challenges, and open questions. J Clin Oncol. 2005;23(6):1295–1311.

Figures

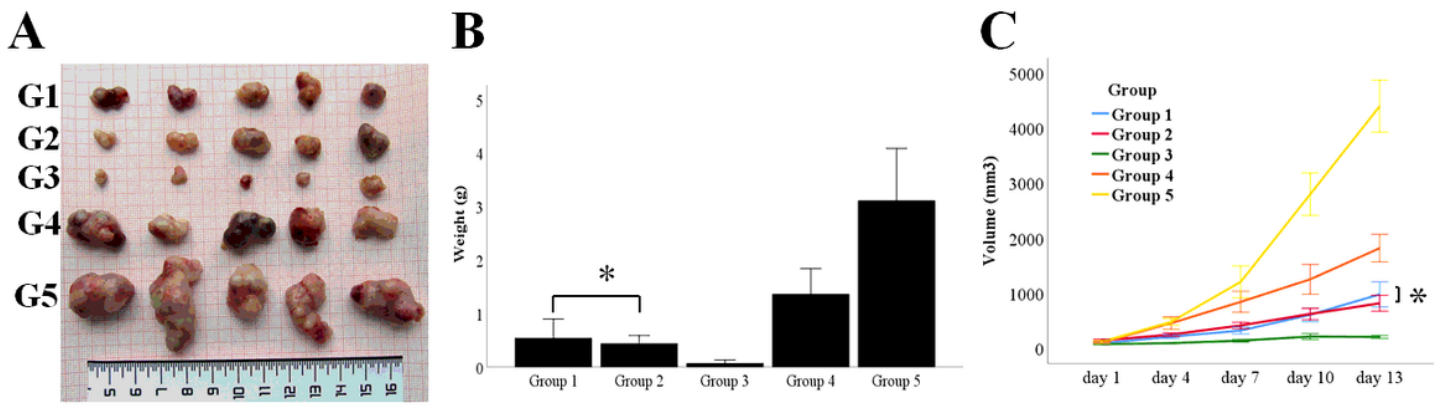

Figure 1

The effects of different interventions on Hepa1-6 tumors in mice. (A) Representative images of different sized tumors from mice in each experimental group. (B) Comparison of tumor weights in five groups. (C) Average tumor volumes growing trend among different treatments. The tumor volumes were determined by calipers at the indicated time points. Data were shown in mean ± SD. * P > 0.05 between two groups shown by a horizontal line and there was a statistically significant difference between any other two groups. G1: TM single treatment; G2: lenvatinib single treatment; G3 combined treatment with TM and lenvatinib; G4: control group; G5: copper-treated group.

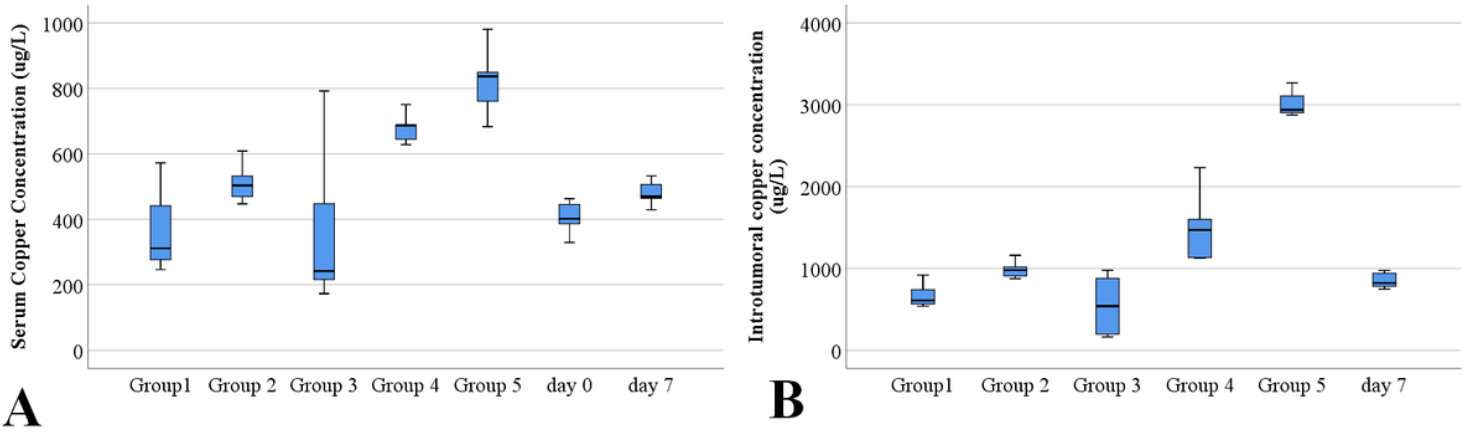

Figure 2

Copper analysis in the serum and tumors. Error bar depicting serum (A) and intratumoral (B) copper concentrations in Hepa 1-6 tumor mice. Copper concentration was compared among mice with different interventions using ICP-MS. The line within each box represents the median; the whiskers represent the maximum and minimum values. Group 1: TM single treatment; Group 2: lenvatinib single treatment; Group 3 combined treatment with TM and lenvatinib; Group 4: control group; Group 5: copper-treated group. Day 0: normal mice without any treatments. Day 7: 7 days after implantation.

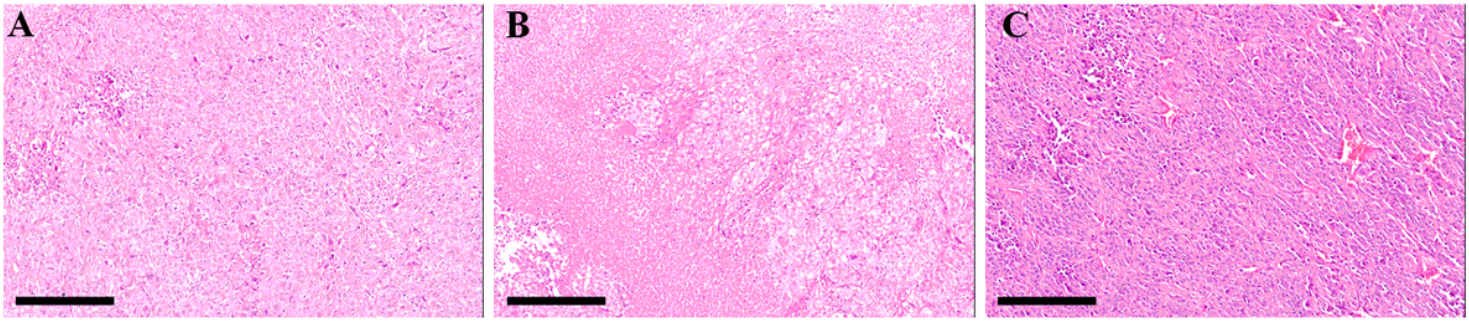

Figure 3

Pathological change of the tumor tissue in each group stained with HE at a magnification of 200×. (A) Control group; (B) TM- and/or lenvatinib-treated group; (C) Copper-treated group. Scale bars indicate 200 μm.

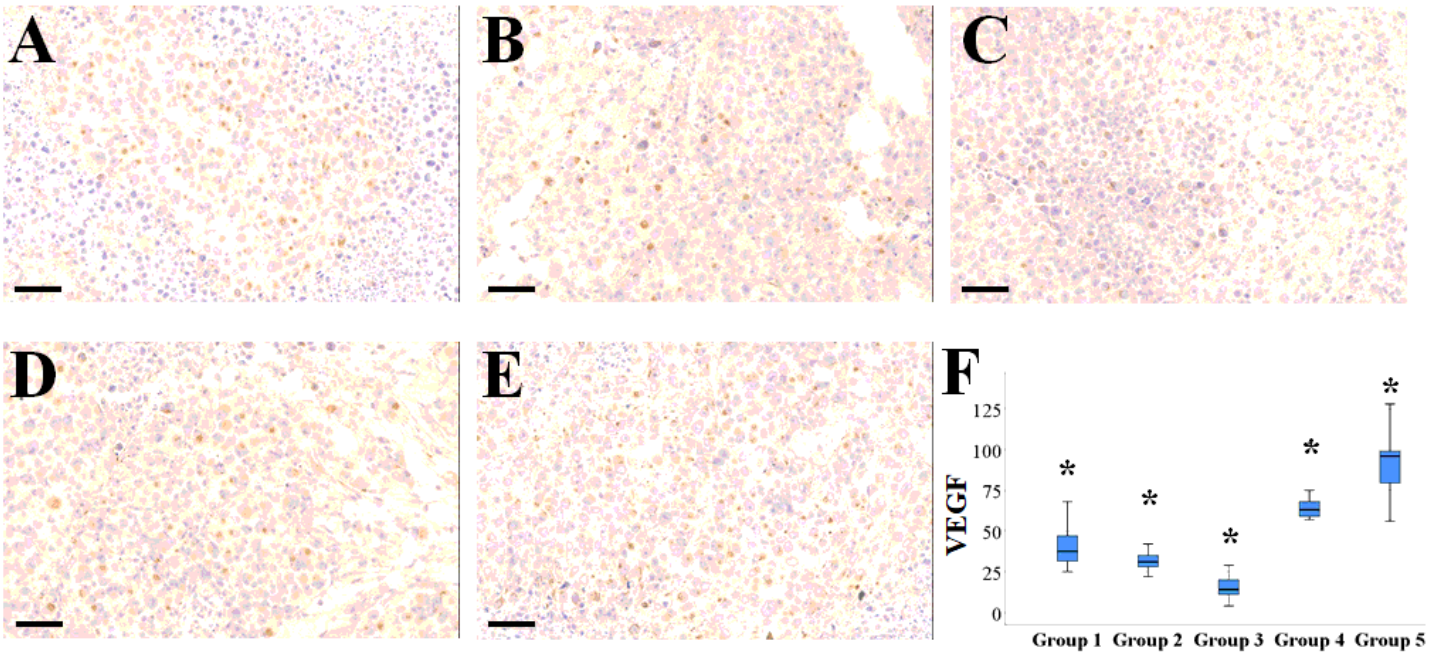

Figure 4

IHC results of VEGF expression in tumor tissue in each group at a magnification of 400×. (A) Group 1: TM single treatment; (B) Group 2: lenvatinib single treatment; (C) Group 3: combined treatment with TM and lenvatinib; (D) Group 4: control group; (E) Group 5: copper-treated group. (F) Expressions of VEGF in the five groups. The whiskers represent the maximum and minimum values. * $P < 0.05$ between every two groups. Scale cars indicate 50 μm.

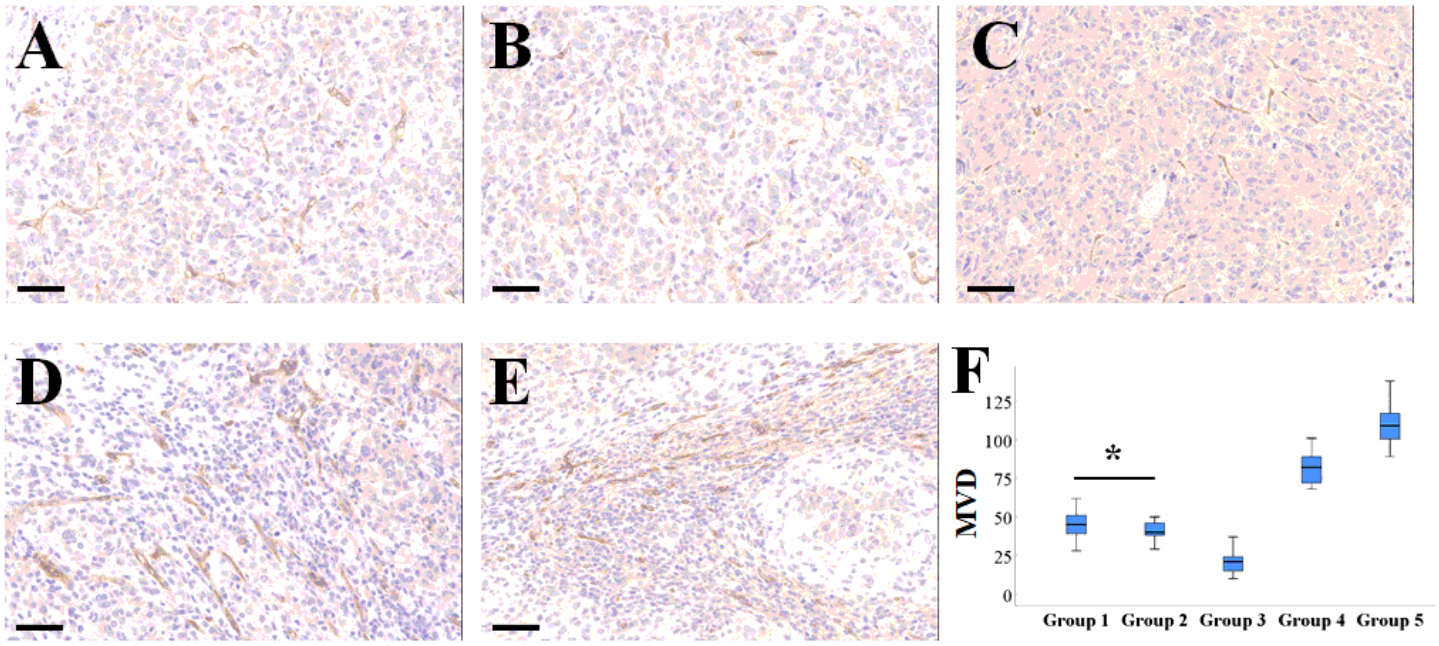

Figure 5

The number of CD34-positive micro-vessels of tumors among different groups detected by IHC. Microvessels were stained as brown. (A) Group 1: TM single treatment; (B) Group 2: lenvatinib single treatment; (C) Group 3: combined treatment with TM and lenvatinib; (D) Group 4: control group; (E) Group 5: copper-treated group. (F) Expressions of MVD in the five groups. * $p > 0.05$ between the two groups shown by horizontal line while significant differences between any two other groups occurred. Scale cars indicate 50 μm. The whiskers represent the 5th to 95th percentile range.