Radiofrequency Ablation Combined with Lenvatinib Improves Ablation Effectiveness in Hepatocellular Carcinoma Mouse Models

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Abstract

Background Hepatocellular carcinoma (HCC) is the second most common cause of cancer-related death. Radiofrequency ablation (RFA) offers unique advantages in the treatment of small HCCs (< 3 cm). The combination of RFA with systemic therapy may have additional benefits for patients. However, there is currently little evidence on the efficacy of RFA combined with Lenvatinib.

Methods SMMC-7721 (human HCC) was implanted subcutaneously into 32 male BALB/c athymic nude mice. The mice were randomly assigned to one of four groups when the tumor volume reached 400-500 mm^3. In the first group, 8 mice received RFA, and the size of their tumors was observed for 12 days. In the second group, 8 mice received Lenvatinib 20 $mg•kg^{-1}•d^{-1}$ by oral gavage for 12 days. In the third group, 8 mice received RFA treatment, followed by 12 days of Lenvatinib treatment. In the fourth group, 8 mice received the solvent only. The RFA output power was 5W for 5 seconds. The ablation effect was visualized by using 2% triphenyltetrazolium chloride (TTC) for 30 minutes and quantified with ImageJ software. HepPar-1, CK18 and Ber-ep4 markers were used for immunohistochemistry. HE staining was used to show tumor tissue morphology and pathological classification. CD31 was used for immunofluorescence to detect microvascular density (MVD).

Results After the 12-day observation, the mean tumor volume in the control group reached 2203.2±173.77 mm3 (standard deviation). The tumors in the mice, grouped by RFA, Lenvatinib and combined treatment, measured 1403.2±589.7 mm^3 ($p<0.05$*), 1014.2±124.03 mm^3 ($p<0.0001$****) and 460.3±86.87 mm^3 ($p<0.0001$****), respectively. The residual tumor active tissue area (%) of the treated groups was 58.64±17.589 ($p<0.001$***), 61.18±3.885 ($p<0.0001$****) and 25.43±5.329 ($p<0.0001$****), respectively, compared with 95.26±1.873 in the control group. Likewise, significant decreases in MVD were noted in the combined treated animals.

Conclusions RFA combined with Lenvatinib facilitated the anti-tumor effectiveness of RFA in the HCC mouse models. This effect may be caused by Lenvatinib reducing tumor blood vessel density and therefore minimizing the heat sink effect.

Keywords Hepatocellular carcinoma, Radiofrequency ablation, Lenvatinib.

Background

Radiofrequency ablation (RFA) is considered as a curative treatment for malignant liver tumors less than 3 cm in diameter [1] and is an important treatment option for patients with advanced or unresectable hepatocellular carcinoma (HCC) [2]. The advantages of RFA include its low morbidity, quick recovery and repeatability. However, the possibility of residual tumor tissue and recurrence present major challenges [3]. The underlying mechanism of residual tumor tissue and recurrence following ablation is thought to involve heat dissipation by large or abundant blood vessels close to the tumor (the heat sink effect) [4]. Therefore, reducing the extent of tumor blood vessels before ablation is hypothesized to significantly improve the RFA efficacy with the goal of achieving complete coagulative necrosis. Indeed, some studies have shown that the tumor response to RFA combined with the pringle maneuver or transarterial chemoembolization (TACE) is associated with superior anti-tumor effect versus RFA monotherapy [5]. However, RFA-TACE combination therapy appears to result in more patient discomfort, longer hospital stays and more frequent complications than RFA or TACE monotherapy [5], which limits the clinical applications of this approach.

Sorafenib is a small molecule tyrosine kinase inhibitor that inhibits tumor angiogenesis and can decrease tumor proliferation. It has been a recommended first-line systemic treatment for advanced liver cancer since 2007 [6]. Both pre-clinical and clinical studies have shown that RFA combined with sorafenib demonstrates better efficacy than RFA monotherapy [7-9]. However, limitations of sorafenib include a modest long-term survival rate and adverse reactions including hand–foot skin reaction, rash, upper and lower gastrointestinal distress, fatigue and hypertension [10]. More recently, Lenvatinib, which is a small molecule inhibitor for vascular endothelial growth factor receptor (VEGFR) 1, 2 and 3, fibroblast growth factor receptor (FGFR) 1, 2, 3 and 4, platelet-derived growth factor receptor alpha, c-Kit and the RET proto-oncogene, has been included in international guidelines as a first-line treatment option for HCC based on the results of the Phase III REFLECT trial [11]. The primary results of REFLECT showed that Lenvatinib is non-inferior to sorafenib for overall survival in the treatment of unresectable HCC, with a lower incidence of hand–foot skin reaction and rash.

Although Lenvatinib combined with RFA may be expected to lead to improved anti-tumor effectiveness for the treatment of HCC, no such data have been published to date. Thus, the purpose of this study was to determine whether RFA combined with Lenvatinib facilitated the anti-tumor effectiveness of RFA in the HCC mouse models.

Methods

Animals

Male BALB/c athymic nude mice, aged 4-6 weeks and weighing 18-20 g were used, sourced from Zhejiang Chinese Medical University (Zhejiang, China). All animal experiments were approved by the Animal Experimentation Committee of Zhejiang University. All experiments were performed in accordance with relevant guidelines and regulations.

Cell Line and Cell Culture

The SMMC-7721 cell line was purchased from China Typical Species Preservation Center

(Shanghai). Cells were cultured in DMEM medium containing 10% fetal bovine serum and placed in a 37℃-incubator containing 95% air and 5% CO2.

HCC and grouping animal models

SMMC-7721 cells (approximately 5×10^6 cells/100 μl) were injected subcutaneously into the right oxters of nude mice, yielding a single tumor per mouse. When the implanted tumor had grown to 400-500 mm^3, the mice were randomized into 4 groups: Control, RFA, Lenvatinib 20 $mg•kg^{-1}•d^{-1}$ by oral gavage, RFA combined with Lenvatinib.

Immunohistochemistry and HE staining

The tumor tissues were identified by immunohistochemistry and HE staining. The immunohistochemistry antibodies included HepPar-1(heptocyte paraffin1), CK18 (Anti-Cytokeratin 18 antibody) and Ber-ep4. Ber-ep4 was used as the negative control.

Drug preparation

Lenvatinib (4 mg) was suspended in 1000μl ddH_2O. The control group mice were treated with the solvent only.

Assessing the efficacy of each treatment

After treatment initiation, the feeding patterns and activity of the mice were observed daily. Tumor size was measured every 3 days. The tumor volume was calculated according to the following formula: $(L\times W^2)/2$, where L represents the maximum length of the tumor and W represents the minimum length of the tumor.

RFA

When the implanted tumor had grown to 400-500 mm3, the RFA group was treated with RFA and then observed for 12 days. The Lenvatinib group and the combined treatment group that had received RFA treatment were observed and received 12 days of Lenvatinib treatment. A 150W radiofrequency generator (MEDSPHERE, Medical Technology Co., Ltd Shanghai, China) was used to apply conventional RFA. Following anesthetic administration (sodium pentobarbital 60 mg/kg), an RFA needle with a 0.5 cm active-tip electrode was used to puncture the tumor. The output power was 5W for 5 seconds.

Assessment of necrosis

After 12 days of observation/administration, the mice were euthanized by cervical vertebra dislocation. Slices were taken at the largest diameter of the tumor and immersed in 2% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma-Aldrich, Germany) for 30 minutes. Areas with mitochondrial enzyme activity in tissues showed red, while the damaged areas were shown as white. The residual tumor active tissue area (%) was quantified with ImageJ software.

Microvascular density assessment

Microvascular density (MVD) was assessed in all groups. Tumor slices of 2-3 mm thickness were incubated with primary antibodies against CD31 (ab28364, Abcam) at 4°C overnight, followed by another 1 hour at room temperature with Cye3-conjugated secondary antibody (GB21303,

Goodbio Technology Co., China). Nuclei were stained with DAPI (Sigma-Aldrich, Germany). Images were acquired using a confocal microscope (Zeiss, Jena, Germany).

Statistical analysis

All results are expressed as mean ± standard error unless stated. Evaluation of the statistical significance of intergroup differences was performed using a two-tailed Student's t-test or one-way analysis of variance. Statistical significance defined as a P value of <0.05 and <0.01 was considered highly significant.

Results

Immunohistochemistry and HE staining

Immunohistochemistry demonstrated that: HepPar-1 was granular and localized in cytoplasm (Fig. 1A); CK18 was strongly positive in cancer cells and the interstitial pseudobile duct (Fig. 1B). Ber-ep4 was used as negative control (Fig. 1C). HE staining demonstrated that a large number of cells were arranged in disorder, with obvious atypia, a round or oval shape and nested or flake arrangement. The nucleus is large and of different sizes; the chromatin is thick. The nucleolus is obvious, and pathological mitotic images can be seen. Hepatoma cells infiltrated and grew around the area. They were accompanied by obvious necrosis, mostly located in the center of the tumor as well as rich neocapillaries, which were mostly located at the edge of the tumor (Fig. 1D).

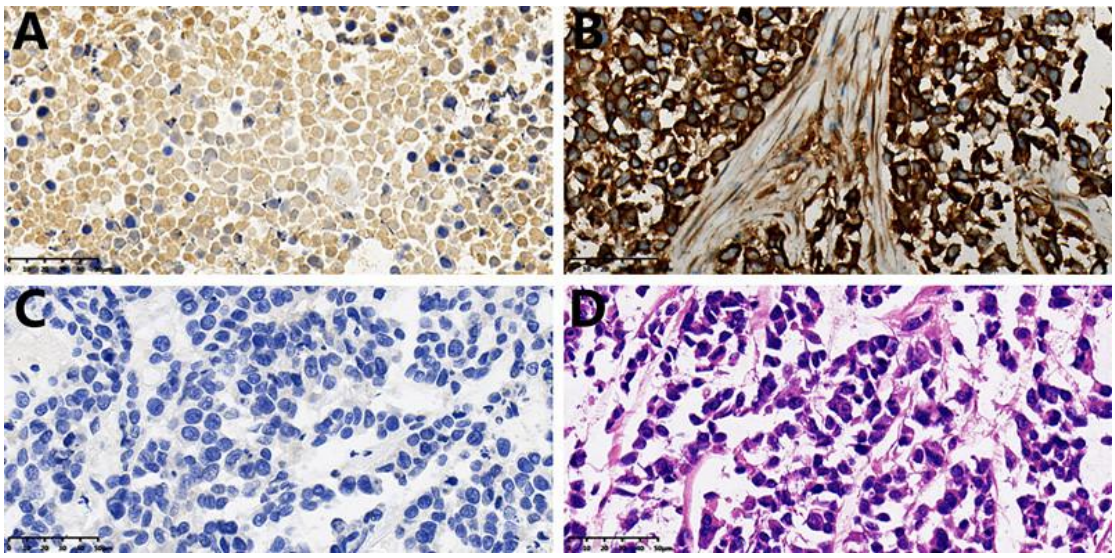

Fig 1. Tumor tissue immunohistochemistry of HepPar-1, CK18, Ber-ep4 and HE staining (Magnification 40×).

The effect of each treatment on tumor volume

After the 12-day administration, the mean tumor volume in the control group reached 2203.2±173.77 mm^3 (standard deviation). Tumors in mice, grouped by RFA, Lenvatinib 20 $mg•kg^{-1}•d^{-1}$ and combined treatment, measured 1403.2±589.7 mm^3 ($p<0.05$*), 1014.2±124.03 mm^3 ($p<0.0001$****) and 460.3±86.87 mm^3 ($p<0.0001$****), respectively. However, the comparison between the RFA group and Lenvatinib group was not statistically significant (P>0.05) (Fig. 2). Our preliminary research data showed that Lenvatinib exerts a concentration-dependent inhibition of HCC growth (supplementary Fig. 1).

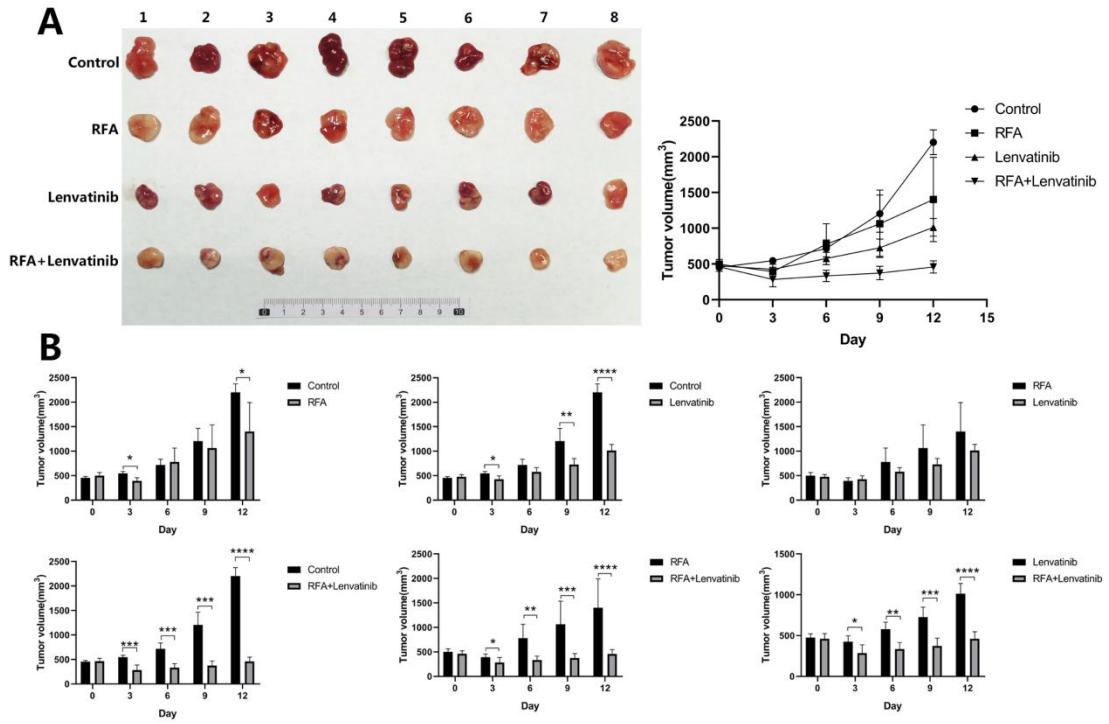

Fig 2. Tumor volume change according to treatment groups. (A) Comparison of tumor volume between treatment groups (RFA, Lenvatinib 20 mg•kg^{-1}•d^{-1}, RFA combined with Lenvatinib) and the Control group. (B) The tumor volume of each treatment group in pairwise comparison. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

The effect of each treatment on radiofrequency ablation

The residual tumor active tissue area was significantly reduced in each treatment group (RFA, Lenvatinib 20 mg•kg^{-1}•d^{-1}, RFA combined with Lenvatinib), compared with the control group. The residual tumor active tissue area (%) was 58.64±17.58, 61.18±3.885 and 25.43±5.329, respectively, versus 95.26±1.873 in the control group (Table 1). The comparison between the RFA group and Lenvatinib group was not statistically significant (P>0.05). This indicated that Lenvatinib may facilitate ablation effect, and the combined effect is more significant (Fig. 3). Our preliminary research data indicated that Lenvatinib exerts a dose-dependent effect on RFA-induced coagulative necrosis of tumors (supplementary Fig. 2).

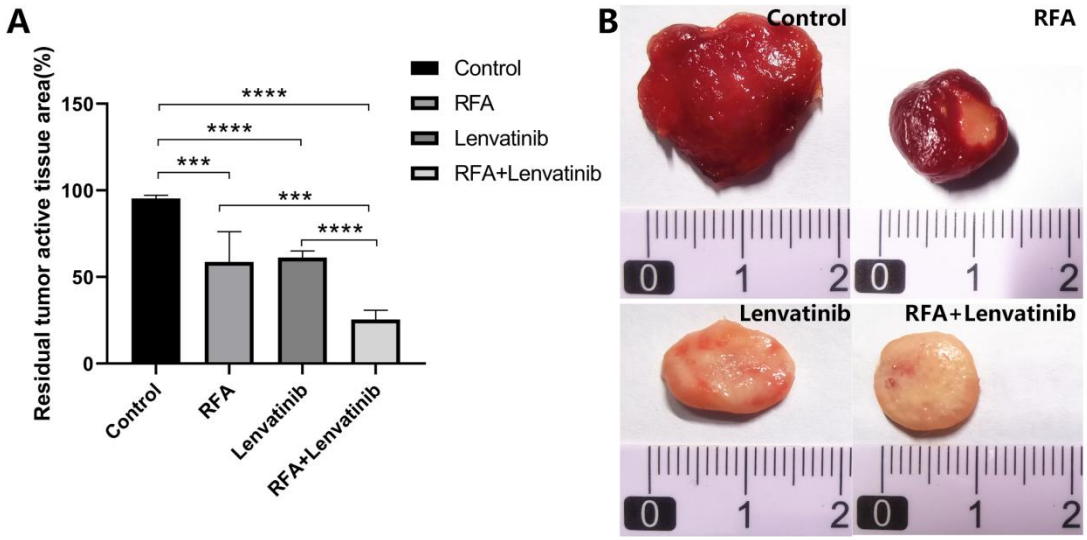

Fig 3. The treatment effect on radiofrequency ablation range. (A) Residual tumor active tissue area, expressed as a percentage: *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. (B) Four representative images of RFA range in the treatment groups (RFA, Lenvatinib 20 mg•kg^{-1}•d^{-1}, RFA combined with Lenvatinib), compared with the control group.

Table 1. Residual tumor active tissue area following different treatments

Grouping	Residual active tissue area %
Control	95.26±1.873
RFA	58.64±17.58***
Lenvatinib	61.18±3.885****
Lenvatinib+RFA	25.43±5.329****

P<0.001.*P<0.0001.

The effect of each treatment on microvascular density

The pharmacological effects of Lenvatinib are known to include inhibition of tumor angiogenesis. Immunofluorescence analysis demonstrated that tumor blood vessel density was decreased in the treatment groups (RFA, Lenvatinib 20 mg•kg^{-1}•d^{-1}, RFA combined with Lenvatinib), compared with the control group. The blood vessel density of the combined treatment group showed a significant decrease (Fig. 4).

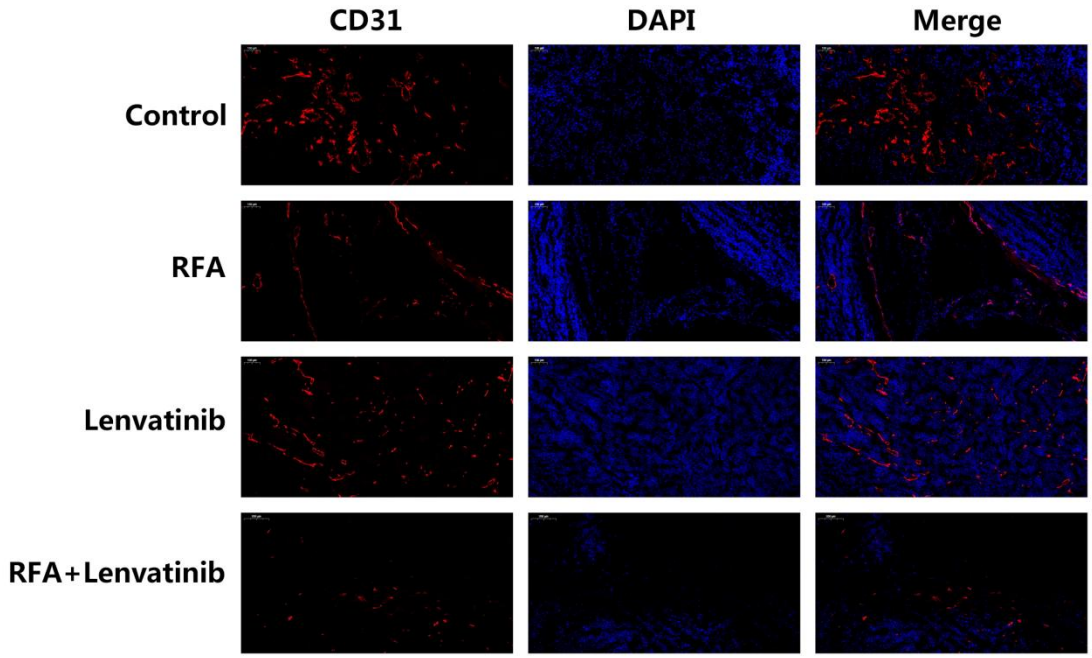

Fig 4. The treatment effect on microvascular density in hepatocellular carcinoma mouse models. CD31 (red staining) was determined by immunofluorescence analysis. Nuclei were stained with DAPI (blue staining). Scale bar, 100 μm.

Discussion

RFA was first used in the treatment of liver cancer in 1996. The basic principle of RFA involves the destruction of tumor tissue via heat energy. The electrode needle sends out electromagnetic waves, which cause the ions and polar macromolecules in the surrounding tissue to vibrate and collide with each other. These waves heat up the tumor area to a specific treatment temperature range for a certain period of time and cause irreversible damage to the tumor cells. This technology has the advantages of being minimally invasive and having fewer complications. It also can result in a shorter hospital stay and protection of liver parenchyma, while also offering repeatability and other advantages. Due to these factors, some liver cancer patients who cannot tolerate surgical resection still have a chance of being treated. Many large-scale clinical studies have demonstrated that the 5-year survival rate of patients receiving RFA can be significantly increased (33%-55%) [12-13].

In order to complete ablation of a tumor, a margin of at least 0.5 cm of surrounding normal tissue is considered to be necessary. The heat-sink phenomenon is an important unfavorable factor that limits the RFA zone size by taking heat away via blood vessels around the tumor [4]. Therefore, we speculate that the inhibition of tumor angiogenesis and blood perfusion would benefit RFA effectiveness.

Lenvatinib is a new multi-target inhibitor for the treatment of unresectable HCC. To our knowledge, this is the first laboratory study to investigate the combination of RFA with Lenvatinib in HCC. Our preliminary research demonstrated that Lenvatinib can inhibit tumor growth and expand the ablation necrotic area in a dose-dependent manner (supplementary Fig. 1 & 2). With

this research foundation, we compared the tumor inhibitory effects of RFA/Lenvatinib alone and in combination. For the combination treated group, we simulated the clinical treatment model: RFA administration was given first, followed by Lenvatinib given by oral gavage. The results indicated that Lenvatinib and RFA exert an inhibition effect on HCC growth respectively, but the most significant reduction was observed in the combined treatment group. Our data demonstrated that Lenvatinib not only exerted an anti-angiogenic effect but also facilitated the ablation effect, which may further inhibit tumor recurrence. Therefore, the combination of RFA with Lenvatinib may be a more effective treatment for HCC than either treatment alone. Relevant clinical trials for this promising treatment mode are currently under way (Table 2).

Table 2. Selected ongoing clinical trials in patients with HCC

Interventions	Enrollment criteria	National clinical trial identifier	Status
RFA; Toripalimab+Lenvatinib	Recurrent HCC	NCT05162898	Not yet recruiting
Lenvatinib; RFA/microwave/electroporation	HCC	NCT05113186	Not yet recruiting
RFA; Tislelizumab/Sintilimab+Lenvatinib/Bevacizumab	Recurrent HCC	NCT05277675	Recruiting
Cryoablation; Sintilimab+Lenvatinib	Liver Metastasis	NCT05098847	Recruiting
Cryoablation; Tislelizumab+Lenvatinib	Advanced HCC	NCT05057845	Recruiting

In conclusion, the results of this study suggest a promising role for combination treatment using RFA with Lenvatinib in HCC. We extrapolate that Lenvatinib may enhance RFA effectiveness through its anti-angiogenic effect.

Author's contributions Zhe Tang designed the experiments. Lantian Wang and Linping Dong performed the experiments and analyzed the data. Bo Zhang, Kezhong Tang, Weifeng Liu and Xin Dong supervised the project. Lantian Wang and Zhe Tang wrote the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval All samples were anonymously coded in accordance with local ethical guidelines (as stipulated by the Declaration of Helsinki) and with written informed consent. The Review Board of the Second Affiliated Hospital of Zhejiang University School of Medicine approved the study protocol.

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