Diagn Interv Radiol 2017; 23:150–155

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INTERVENTIONAL RADIOLOGY

ORIGINAL ARTICLE

Study on the effect of chemoembolization combined with microwave ablation for the treatment of hepatocellular carcinoma in rats

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PURPOSE
We aimed to evaluate the combining effects of transarterial chemoembolization (TACE) and open local thermal microwave ablation in a hepatocellular carcinoma animal model.

METHODS
Tumor cubes were implanted into the liver of 30 male inbred ACI rats. Groups of 10 animals were treated at 13 days (TACE or microwave ablation) and 16 days (microwave ablation) postimplantation with combined therapy of TACE (0.1 mg mitomycin C; 0.1 mg iodized oil; 5.0 mg degradable starch microspheres) and microwave ablation (2450 Mhz; 45 s; 35 W) (study group A), TACE alone (control group B), or microwave ablation alone (control group C). At day 12 and day 25 tumor size was measured via magnetic resonance imaging and the relative growth ratio was calculated. Hepatic specimens were immunohistochemically examined for the expression of vascular endothelial growth factor (VEGF).

RESULTS
Mean growth rates were 1.34±0.19 in group A, 3.19±0.13 in group B, and 4.18±0.19 in group C. Compared with control groups B and C, tumor growth rate in group A was significantly inhibited ($P < 0.01$). The VEGF-antibody reaction in peritumoral tissue (staining intensity at portal triad, percent antibody reaction and staining intensity at central vein) was significantly lower in group A compared with group B ($P < 0.01$). No significant difference between group A and group C could be observed.

CONCLUSION
This investigation shows improved results of TACE followed by microwave ablation as treatment of hepatocellular carcinoma in a rat model, compared with single therapy regimen regarding the inhibition of growth rate and reduction of VEGF-level in peritumoral tissue.

Hepatocellular carcinoma (HCC) is the fifth most common malignancy among men, and the seventh most common malignancy among women. Although morbidity and mortality are very high, no standardized treatment algorithm exists to date (1). Transarterial chemoembolization (TACE) is a frequently used interventional treatment for patients with inoperable HCC (2). Direct application of drugs into tumor feeding branches, allows for higher local chemotherapeutic concentrations with less systemic side effects. However, TACE has not been shown to notably prolong the overall survival rate, mainly due to local and distal tumor recurrence after treatment (3). Microwave ablation is a minimally invasive technique with low complication rate for the nonsurgical treatment of HCC (4). Electromagnetic waves at frequencies of 900–2450 MHz are used to induce coagulation necrosis. Polar molecules (mainly water dipoles) try to realign themselves with the direction of current in an electromagnetic field. The oscillation produced by constant realignment causes friction and a heating effect. However, it has been shown that local tumor control is highly dependent on complete tumor ablation, and recurrence in larger nodules (> 5 cm in diameter) is remarkably worse than in smaller ones (5, 6).

Recently published studies imply that a combination of TACE and local ablation might have a synergistic effect on HCC (7). TACE leads to inflammatory edema, therefore increasing the watery content of the tumor, which the technique of microwave ablation uses to produce heat (8). Furthermore, central tumor parts in close vicinity to the probe receive the greatest thermal insult during physical ablation, while TACE works best on the better vascularized peripheral tumor areas (3). Trials with over 100 patients demonstrated that combined therapy of microwave ablation applied within six weeks after TACE can effective-

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Received 29 January 2016; revision requested 8 April 2016; last revision received 18 July 2016; accepted 26 July 2016.

Published online 10 February 2017.
DOI 10.5152/dir.2016.16617

ly treat HCC, even those tumors larger than 10 cm in dimension. Compared with single treatment regimen, the combination therapy yielded improved results regarding complete necrosis rate, tumor growth reduction and overall survival (9, 10). However, the effect of microwave ablation after TACE on the hypoxic cells and subsequent vascular endothelial growth factor (VEGF) production is unclear. Moreover, the mechanism of this synergetic effect is not known.

Therefore, the aim of this investigation was to evaluate the growth inhibiting effect and influence on histopathologic changes of growth factor production of combined TACE-microwave ablation treatment of HCC in a small animal model.

Methods

Animals and tumor implantation

Veterinary department approval was obtained and experiments were performed according to their guidelines. Morris hepatoma 3942A (CLS Cell lines service GmbH), which is similar to human hepatocellular carcinoma, was implanted in August Copenhagen Irish rats (ACI, Harlan Sprague Dawley Inc.) in this study. The used cell line represents a high grade, poorly differentiated HCC with a rapid growth rate. The inbred male rats were thirteen weeks old and weighed about 230 g. Animals had unrestricted access to food and liquid in a standardized constant environmental condition.

During all surgical and diagnostic procedures, laboratory animals were anesthetized with intraperitoneal injections of ketamine (Ketanest, Pfizer; 100 mg/kg) and xylazine (Rompun, Bayer AG; 15 mg/kg).

Main points

- Combination therapy of transarterial chemoembolization (TACE) and microwave ablation might have a synergistic effect on the treatment of hepatocellular carcinoma.
- Based on our rat hepatocellular carcinoma model, the mean tumor growth ratios were smaller in the TACE-microwave ablation combination group than the control groups, treated with either TACE or microwave ablation alone.
- After treatment with TACE, a lesser VEGF-expression in peritumoral tissue could be observed in TACE-microwave ablation combination group than in TACE alone group.
- The excessive production of growth factors by tumor cells triggered by ischemia after TACE could be reduced by a second intervention with a different treatment modality.

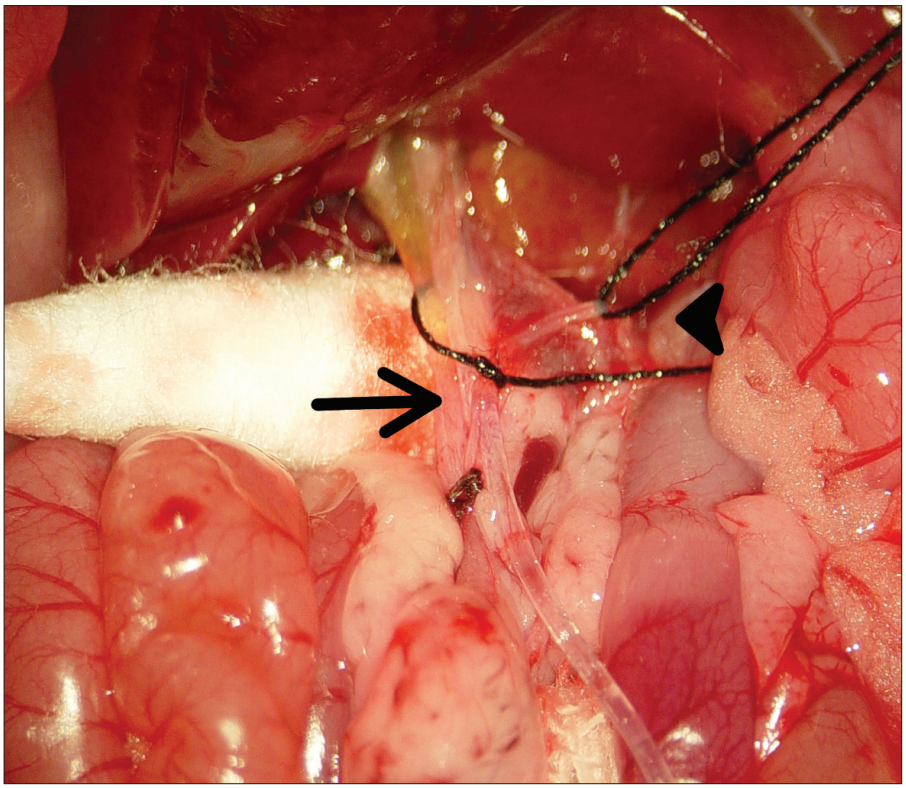

Figure 1. For transarterial chemoembolization (TACE) (mitomycin, iodized oil, starch microspheres) a polyethylene microcatheter (diameter, 0.61 mm) was inserted into the gastroduodenal artery and the nozzle was advanced to the proper hepatic artery *(arrow)*. A pre-placed silk suture prevented retrograde drug flow into the common hepatic artery *(arrowhead)*.

We used a slightly altered tumor implantation technique according to the description by Yang et al. (11, 12). Tumor suspensions (approximately 5×10^6 tumor cells) were injected subcutaneously into the flank of three donor animals. After incubation time of 12 days, tumors were recovered and vital parts were split into masses with a diameter of approximately 1.25 mm and volume of 2 mm^3. On day 0, the abdominal cavity of laboratory animal was exposed and through an incision of the left liver lobe a tumor piece was inserted beneath the liver capsule. After bleeding control with cotton swaps, the abdomen was closed.

Interventional therapy

Interventional therapies were given on day 13 and day 16. Study animals were divided into three groups and each group was treated according to the following sequences:

- Study group A (TACE + microwave ablation, n=10): mitomycin C (0.1 mg) + iodized oil (0.1 mL) + degradable starch microspheres (5.0 mg) on day 13 and microwave ablation (2450 MHz, 35 W, 45 s) on day 16.
- Control group B (TACE alone, n=10): mitomycin C (0.1 mg) + iodized oil (0.1 mL) + degradable starch microspheres (5.0 mg) on day 13.
- Control group C (microwave ablation alone, n=10): microwave ablation (2450 MHz, 35 W, 45 s) on day 13.

TACE: The abdominal cavity was opened by midline incision and the hepatic vascularization was uncovered. The liver supplying vessels were surgically dissected and exposed. Magnified by a surgical microscope (OP-Mi-6, Zeiss), a polyethylene microcatheter (Portex PE-10) was introduced into the gastroduodenal artery and the nozzle advanced to the proper hepatic artery. Drugs were administered through the catheter into the liver (subsequent injection of mitomycin C (Roche) + iodized oil (Lipiodol, Guerbet GmbH) + degradable starch microspheres (Embocept, Pharmacept) within 20 minutes. After removal of the catheter, the gastroduodenal artery was ligated with a pre-placed silk suture (Fig. 1).

Microwave ablation: A small subxiphoid laparotomy was performed and the liver lobe bearing the implanted tumor was retracted out of the abdominal cavity. A needle antenna (AMICA-Probe, 16 G × 150 mm; HS Hospital Service) with a mini-choke and internal water cooling was used. The antenna was connected to a generator (AMICA-GEN 3.0; HS Hospital Service), operating at a microwave frequency of 2450 MHz. A power of 35 W was applied for 45 s, according to the recommendation of the manufacturer. The anticipated burn size diameter was expected at 5–8 mm (13). Tumors could be visually identified due to their superficial, immediate subcapsular position; correct insertion site of the probe was additionally confirmed through palpation. Coagulation was deemed satisfactory when the tissue became yellow or light brown in color. For bleeding control, gelfoam strip (Pharmacia & Upjohn Co.) was used to fill the needle tract after microwave ablation (Fig. 2).

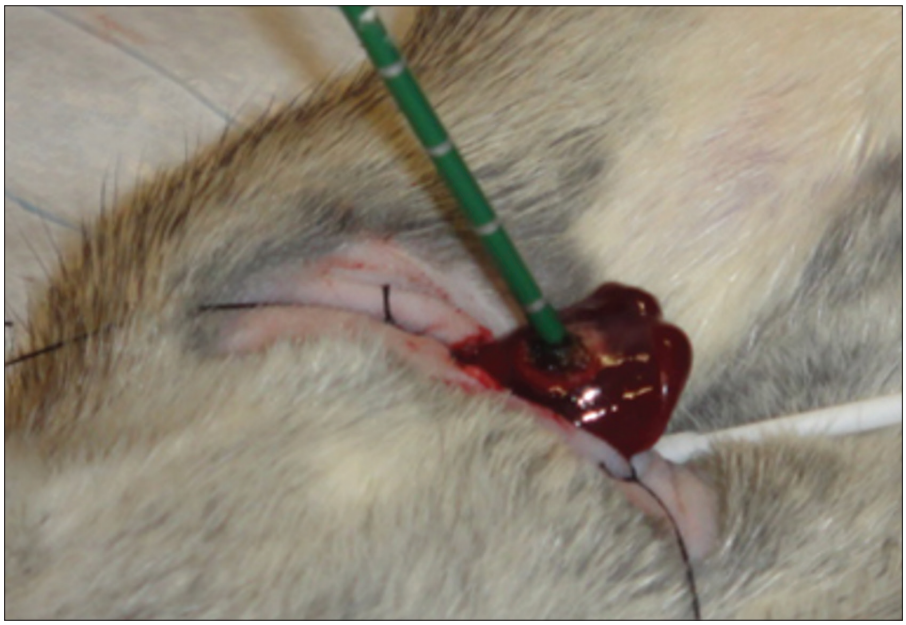

Figure 2. For microwave ablation a needle antenna was placed in the middle along the longest diameter of the tumor after laparotomy. The connected generator uses microwave frequency of 2450 MHz and applies a power of 35 W for 45 seconds.

Magnetic resonance imaging

Magnetic resonance imaging (MRI) scans were performed prior to treatment (day 12) and at the end of the observation period (day 25) on a 3.0 T MRI unit (Magnetom Trio, Siemens) using a multipurpose coil (CPC 8-Ch Multipurpose coil; NORAS MRI products). T1-weighted (Spin-echo: TR/TE, 500/12 ms) and T2-weighted (Turbo spin-echo: TR/TE, 3870/80 ms) transverse images with a section thickness of 2 mm and 192×256 matrix were acquired. The entire tumor volume, including intratumoral hypointense area after treatment, was calculated from T2-weighted images using the formula (14): $V=1/2*d1*d2^2$ (d1, largest tumor diameter; d2, diameter perpendicular to d1).

Immunohistochemical examination

On day 26, explanted tumor specimens were macroscopically inspected for vital tumor parts and necrosis extension. Samples were embedded in Tissue-Tek (Sakura), frozen at -80° C, and eventually sliced into 5 µl

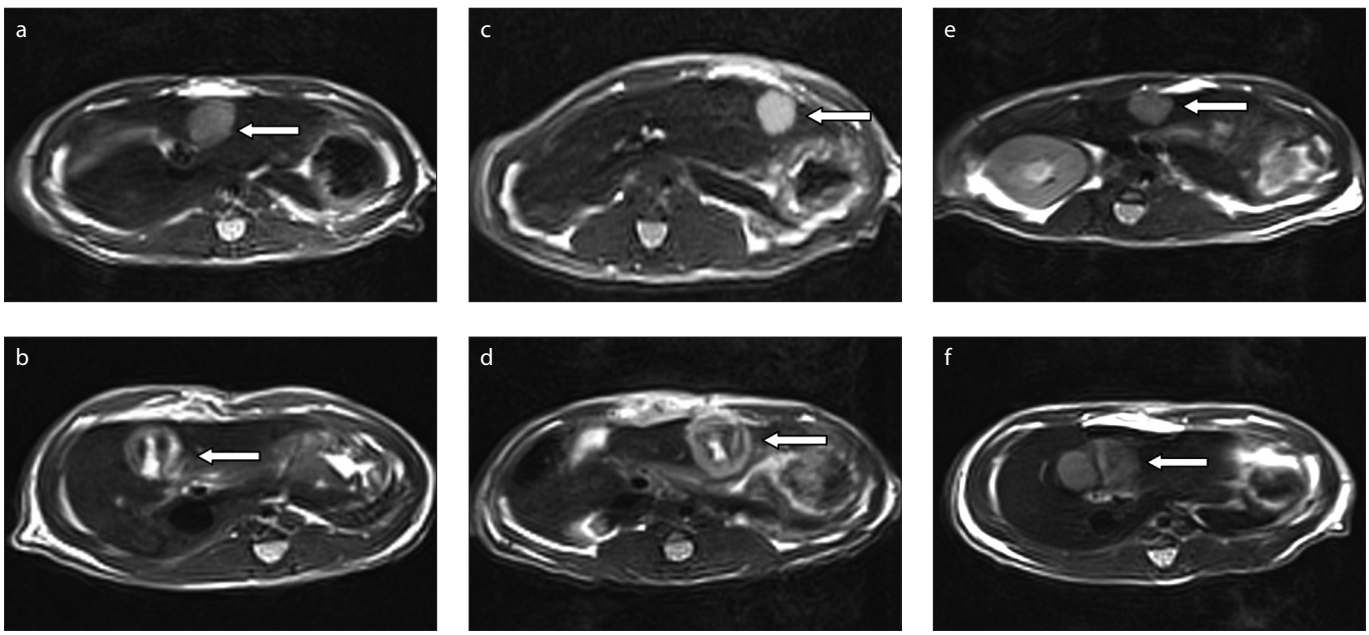

Figure 3. a–f. Representative transverse unenhanced T2-weigthed turbo spin-echo MRI of liver tumors in study group A (TACE-microwave ablation, **a** and **b**), control group B (TACE alone, **c** and **d**) and control group C (microwave ablation alone, **e** and **f**) in ACI rats. Pretreatment image **(a)** from group A shows a hyperintense tumor (*arrow*, 0.90×0.75 cm). Post-treatment image **(b)** of the same lesion (*arrow*, 1.10×0.75 cm) shows hyperintense tumor characterized by an inhomogeneous hypointense area corresponding to intratumoral coagulation. Pretreatment image **(c)** from group B shows a hyperintense tumor (*arrow*, 0.75×0.70 cm). Image **(d)** depicts the same tumor (*arrow*, 1.03×0.87 cm) post-treatment; it is bigger in size, indicating rapid tumor growth. Pretreatment image **(e)** from group C shows a hyperintense tumor (*arrow*, 0.64×0.45 cm). After thermal ablation, image **(f)** shows a larger tumor (*arrow*, 1.30×0.72 cm) indicating rapid growth and a central hypointense area.

Table. Immunoexpression of VEGF in tumor and surrounding tissue

		TACE+microwave ablation			TACE alone				Microwave ablation alone			
		Median	Min	Max	Median	Min	Max	*P*	Median	Min	Max	*P*
Tumor	Percentage	5	0	5	5	1	5	0.2909	5	2	5	0.7687
	Intensity	2	0	3	2	1	3	0.4793	2	1	3	0.1263
Portal triad	Percentage	2	0	4	2	0	4	0.0952	2	0	3	0.6648
	Intensity	1	0	3	2	0	3	0.0092*	1	0	3	0.5155
Central vein	Percentage	0	0	3	1	0	4	0.0038*	1	0	3	0.6043
	Intensity	0	0	2	1	0	3	0.0014*	1	0	2	0.5965

Scale for staining percentage: 0 (no staining), 0 (no staining), 1 (≤5%), 2 (6%-25%), 3 (26%-50%), 4 (51%-75%), 5 (≥76%).
Scale for staining intensity: 0 (no staining), 1 (low intensity), 2 (intermediate intensity), 3 (high intensity).
VEGF, vascular endothelial growth factor; TACE, transarterial chemoembolization; Min, minimum; Max, maximum.
*Significant results compared with group TACE+MWA ($P < 0.05$, Wilcoxon signed rank test).

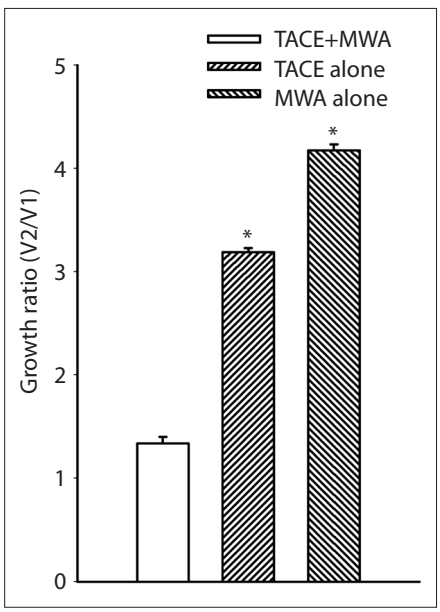

Figure 4. The mean tumor growth ratio (V2/V1) calculated from MRI scans in each group. Compared with groups B (TACE alone) and C (microwave ablation [MWA] alone), group A (TACE-MWA) showed significant reduction in tumor growth ($P < 0.01$, Bonferroni test).

cryosections. Sections were incubated with anti-VEGF rabbit polyclonal antibody (Santa Cruz Biotechnology). Anti-rabbit alkaline phosphatase supervision polymer system (DCS Innovative Diagnostik-Systeme) was used as a secondary antibody. Sections were stained with new Fuchsin substrate chromogen (DCS Innovative Diagnostik-Systeme) and hematoxylin. Growth factor expression was assessed by two observers, blinded to treatment and imaging data. The final score was reached by consensus. Stained cells were counted in 5 microscopic fields (×100) per slide in tumor, peritumoral tissue defined as portal triad, as well as central vein area. Slides were evaluated by a semiquantitative method measuring the percentage staining of the cells and were recorded ranging from 0 to 5 (0, no staining; 1, ≤5%; 2, 6%–25%; 3, 26%–50%; 4, 51%–75%; 5, ≥76%). In addition, positively stained slides were evaluated for staining intensity and recorded from 1 to 3 (1, low; 2, intermediate; 3, high).

Statistical analysis

The tumor growth rate (V2/V1, V2 tumor volume at end of observation period, and V1 tumor volume before treatment) was measured by MRI, and the differences between the treatment modalities were analyzed using the statistical software GraphPad Prism (v. 4.03).

Immunohistochemical data (staining percentage and intensity of VEGF expression) was evaluated using descriptive and semiquantitative methods. Analysis for normal distribution was performed using the Kolmogoroff-Smirnoff-Lilliefors test. Comparisons between groups were made using the Bonferroni or the Mann-Whitney U test using BiAS (v. 10.0, University Hospital Frankfurt).

Differences with a *P* value less than 0.05 were considered statistically significant.

Results

The HCC model attained a highly successful tumor generation rate (100%). On day 13 of the experimental phase, unenhanced MRI was able to detect a mass in the left lateral hepatic lobe of all 30 animals.

In group A rats subjected to combined TACE-microwave ablation therapy, post-treatment T2-weighted MRI showed a solid hyperintense tumor with centered hypointense coagulation (Fig. 3a, 3b). In group B rats subjected to TACE alone (Fig. 3c, 3d) and group C rats subjected to microwave ablation therapy (Fig. 3e, 3f), a relatively accelerated growth rate was observed. Furthermore, MRI of group C showed incomplete intratumoral coagulation with a hypointense motive.

Mean tumor size was 132.30±79.91 mm^3 prior to treatment: group A, 182.93±93.36 mm^3; group B, 104.20±78.84 mm^3; group C, 109.77±36.84 mm^3, with no significant difference between groups except for borderline significance between groups A and C, $P = 0.033$. Post-treatment mean tumor sizes were 234.77±111.79 mm^3, 324.78±232.42 mm^3 and 456.69±150.09 mm^3, for groups A, B, and C, respectively. The mean tumor growth ratios (V2/V1) were 1.33±0.19, 3.19±0.13, and 4.17±0.18, for groups A, B, and C, respectively. Tumor growth rate was significantly reduced in study group A compared with control groups B and C ($P < 0.001$; Fig. 4).

Morphology of liver tumor showed that the combined therapy increased the central coagulation of the tumor, and a thin layer was observed around the tumor (Fig. 5a). Rats treated with TACE alone, showed relatively smaller necrotic area in the middle of the tumor with a much thicker layer around the central zone (Fig. 5b). The treatment with microwave ablation alone led to increased central coagulation of the tumor; however, an enlarged tumor rim could also be observed (Fig. 5c). Microscopically those areas could be identified as vital tumor parts, expressing growth factor, and separating the central necrotic parts from normal liver tissue. Growth factor expression of tumor was evaluated using anti-VEGF antibodies. All tumor specimens showed VEGF expression. The expression of these proteins was confirmed by immunohistochemical analysis showing a red-brown

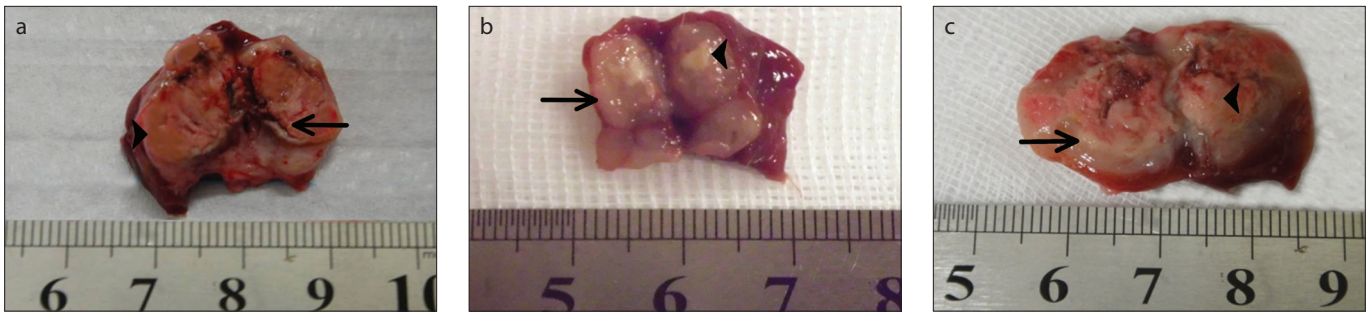

Figure 5. a–c. Morphology of liver tumor in ACI rats. In group A, combined therapy with TACE-microwave ablation **(a)**, led to increases in the central coagulation of the tumor (yellow zone, *short arrow*); a thin white layer is observed around the tumor *(long arrow)*. In group B, rats treated with TACE alone **(b)** show relatively smaller necrosis in the middle of the tumor *(short arrow)*. In group C, rats treated with microwave ablation alone **(c)** show increases in the central coagulation of the tumor *(short arrow)*, but a thicker white layer can be observed around the central zone *(long arrow)*.

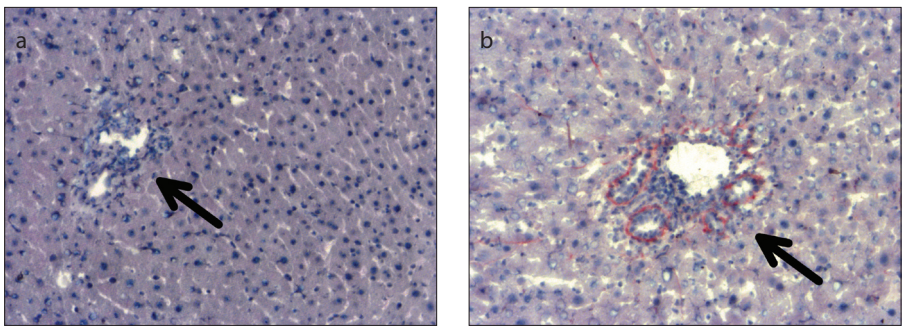

Figure 6. a, b. Immunohistochemical anti-VEGF-staining of a specimen of **(a)** group A (TACE-microwave ablation) and **(b)** group B (TACE alone) (×100). Higher scores on staining percentage and intensity on average can be observed in group B around the portal triad *(arrows)* in comparison to group A.

cytoplasmic staining within the cells. Significantly lower VEGF-antibody reaction in peritumoral tissue was observed in group A compared with group B ($P = 0.009$ for staining intensity at the portal triad, $P = 0.004$ for percentage antibody reaction, and $P = 0.001$ for staining intensity at the central vein; Table and Fig. 6a, 6b); however, no significant differences were observed between groups A and C.

Discussion

HCC is a highly malignant primary tumor of the liver that accounts for almost 750,000 deaths per year worldwide (1). Results of recent studies suggest that TACE and microwave ablation can supplement each other synergistically when treating HCC (7).

The purpose of this experimental investigation was to determine whether combined TACE-microwave ablation can lead to better outcomes than TACE or microwave ablation alone in rat models of HCC and to investigate differences in growth factor expression in histologic specimens.

In this study, tumor growth rate was significantly reduced with combined treatment of TACE-microwave ablation compared with single treatments of TACE or microwave ablation. We refrained from a third control group with no treatment (e.g., transarterial saline infusion only), because vastly increased tumor growth rate could be expected based on the results of a previous study (12). We also determined that vessels surrounding the tumors had significantly weaker VEGF expression following combined therapy in group A compared with TACE alone treatment in group B.

The interventional treatment modalities of Morris hepatoma in ACI rats are comparable to that of human HCCs with a promising outcome (3, 15). The feasibility of this combined treatment between TACE and physical ablation with laser-induced thermotherapy has been proven by a previous experiment in an animal model with liver metastases (16).

VEGF-antibody reaction in peritumoral tissue was not significantly different between the combined therapy and microwave ablation therapy groups, although the growth rate difference is highest between those two groups. We assume that other cytokines (e.g., liver basic fibroblast growth factor, transforming growth factor-β 1) might have a greater impact on the further development of tumors, following possible subtherapeutic thermal ablation and absence of an ischemia-inducing treatment (17). Similar findings have been observed in past experiments with comparable treatment modalities in small animal setups as well (16).

Different clinical trials demonstrated that combined therapy of microwave ablation applied within six weeks after TACE can effectively treat HCC. Compared with single treatment regimen, the combination therapy yielded improved results regarding complete necrosis rate, tumor growth reduction, and overall survival (8, 9, 18). Combined therapy might have a synergistic effect for the following reasons: microwaves produce thermal energy through dielectric heating of water molecules in the affected tissue. TACE reduces the hepatic blood flow and therefore the cooling effect by convection during the ablation process (19). Furthermore, ischemic and inflammatory effects of TACE therapy can induce edematous change in the tumor and peritumoral parts of the liver, and therefore may enlarge the coagulation area (8). In addition, microwave ablation acts directly on the center of the tumor, while TACE has more therapeutic effects on the greater vascularized periphery of the tumor.

Our study suggests a further synergistic effect: a successful TACE leads to the occlusion of the tumor feeding vessels and causes hypoxia in the malignancy. Consequently, the level of active hypoxia-inducible-factor increases and raises the production of VEGF, which has also previously been demonstrated in animal and human studies (20, 21). A follow-up intervention with different treatment modality, like microwave ablation in this case, might be able to impair ischemia-triggered tumor cells before more adverse effects can be caused by excessive levels of VEGF.

The two different interventional treatments of liver cancer seemed to overcome their own deficiencies (e.g., high recurrence rate and limited ablation size) and elicited treatment results, which is impossible to receive from a single treatment approach (3, 5, 6). Microwave ablation after TACE may induce a larger area of complete tissue necrosis than ablation alone, therefore lessening the risk of local recurrence.

Despite careful preparation, there were limitations in our study. First, the morphologic assessment of specimens was performed macroscopically. A microscopic evaluation of the tumor was out of the scope of the study, particularly since macroscopic changes were very evident. Second, relatively small size of the lesions and lack of contrast injection made it very difficult to accurately identify the necrotic area within the tumor, particularly since necrotic and non-necrotic areas had the same signal intensity on T2-weighted imaging. Third, tumors in groups A and C were significantly different in size prior to treatment. However, the tumors in the study group were larger than the tumors in the control groups, hence the challenge to test the method under investigation was larger (22). Finally, the hepatic expression of VEGF has not been measured with more precise quantitative methods such as Western blot to support the findings in the immunohistochemical analysis, because the extraction of specific peritumoral tissue for analysis would have been technically challenging.

In conclusion, the combination of TACE and microwave ablation, compared with TACE or microwave ablation alone, triggered a significant reduction in the growth rate of liver tumors, as well as VEGF level in peritumoral tissue in ACI rats. Our results suggest that further laboratory and clinical investigations of this combined interventional therapy are reasonable in order to develop a promising treatment protocol for patients with unresectable HCCs.

Conflict of interest disclosure

The authors declared no conflicts of interest.

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