

# Combined Radiofrequency Ablation and Double Anti-Angiogenic Protein Therapy to Increase Coagulation Efficacy: An Experimental Study in a Murine Renal Carcinoma Model

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**Objective:** To evaluate whether suppression of tumor microvasculature by double anti-angiogenic protein (DAAP) treatment could increase the extent of radiofrequency ablation (RFA)-induced coagulation in a murine renal cell carcinoma model.

**Materials and Methods:** Renal cell carcinoma cell lines were implanted subcutaneously into 10 nude mice. Four mice received adenoviral DAAP treatment and 6 mice received sterile 0.9% saline solution as DAAP-untreated group. The effect of DAAP was evaluated according to the vascularity by contrast-enhanced ultrasound (CEUS) using microbubbles. Four DAAP-treated mice and 4 DAAP-untreated mice were then treated with RFA, resulting in 3 groups: no-therapy (n = 2), RFA only (n = 4), and RFA combined with DAAP treatment (n = 4). Immediately after RFA, the size of coagulation necrosis and mitochondrial enzyme activity were compared between the groups using analysis of variance (ANOVA) and post hoc test.

**Results:** The contrast enhancement ratio for tumor vascularization on CEUS was significantly lower in the DAAP treated group than in DAAP-untreated group ( $30.2 \pm 9.9\%$  vs.  $77.4 \pm 17.3\%$ ;  $p = 0.021$ ). After RFA, the mean coagulation diameter was 0 mm for no-therapy group,  $6.7 \pm 0.7$  mm for the RFA only group and  $8.5 \pm 0.4$  mm for the RFA with DAAP group (ANOVA,  $p < 0.001$ ). The area of viable mitochondria within the tumor was  $27.9 \pm 3.9\%$  in no-therapy group,  $10.3 \pm 4.5\%$  in the RFA only group, and  $2.1 \pm 0.7\%$  in the RFA with DAAP group (ANOVA,  $p < 0.001$ ).

**Conclusion:** Our results suggest the potential value of combining RFA with anti-angiogenic therapy.

**Index terms:** Radiofrequency ablation; Angiogenesis; Anti-angiogenic protein; Renal cell carcinoma; Mice model

## INTRODUCTION

Renal cell carcinoma (RCC) was diagnosed in more than 88400 patients in Europe in 2008 and caused about 39300 deaths (1, 2). Many cases are incidentally

found during cross-sectional imaging examinations such as ultrasonography (3, 4). Further, there have been major advances in relatively less invasive treatments for RCC, as compared to that in nephrectomy (5, 6). These developments include laparoscopic nephrectomy, and ablation therapies such as cryoablation and percutaneous image-guided radiofrequency ablation (RFA) (7-9).

Recently, RFA was reported to be safer for patients than radical nephrectomy. As a result, RFA has been performed increasingly to treat small tumors, as it provides effective destruction of RCC stage T1a tumors (< 4 cm) (10, 11). However, the major limitation of RFA is incomplete treatment of larger lesions such as RCC stage T1b (> 4 cm).

Several techniques for increasing tumor destruction to aid in RFA have included surgical and angiographic techniques, such as the Pringle maneuver (i.e., vascular clamping of

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portal inflow at surgery) and chemoembolization (12, 13). However, these techniques are disadvantageous since they require invasive procedures, contrary to the purported benefit of minimally invasive therapy. Thus, concomitant administration of adjuvant antivasular pharmaceutical agent such as double anti-angiogenic protein (DAAP) agents that maximize the antivasular effect would be of considerable clinical interest (14, 15). DAAP, which can bind and block both VEGF-A and angiopoietins, is more highly effective than VEGF-Trap alone in inducing tumor vessel regression and reducing subsequent blood flow in the established tumor (16).

Furthermore, the double blockade of VEGF-A and Ang-2 with DAAP showed marked effectiveness in suppressing tumor angiogenesis and metastasis of implanted and spontaneous solid tumors and in reducing ascites formation and vascular leakage in advanced ovarian cancer (14). Accordingly, we hypothesized that combining RFA with double anti-angiogenic therapy has increased ablation efficacy. The purpose of this study was to evaluate whether suppression of tumor microvasculature by DAAP treatment could increase the extent of RF-induced coagulation in a murine RCC model.

## MATERIALS AND METHODS

### Experimental Animals and Generation of Tumor Model

Specific pathogen-free nude mice were purchased from Central Laboratory Animal Inc. (Seoul, Korea), and were bred in our pathogen-free animal facility. Ten male nude mice (aged 5 weeks and weighing 20 g each) were used for this study. All mice lived in a system equipped with day-night light cycling and were provided with standard mouse chow. Experimental procedures were performed with approval from the Animal Care Committee of our institute.

Renal cell carcinoma 786-0 human renal cell carcinoma cells ( $5 \times 10^7$ ) suspended in 0.1-mL phosphate-buffered saline were injected subcutaneously into the unilateral inguinal area of nude mice and were allowed to expand for 14 days until the tumors grew to a size of approximately  $1.0 \text{ cm}^3$ .

### Treatment with DAAP

A recombinant adenoviral vector encoding the DAAP gene was provided by Koh et al. (14). For DAAP treatment, the indicated amount ( $1 \times 10^9$  plaque forming units [pfu]) of diluted adenovirus in 50  $\mu\text{L}$  of sterile 0.9% saline was

injected intravenously through the tail vein approximate 14 days (tumors 1.5–2.0 cm in diameter) after implanting RCC-786-0 in 4 nude mice (DAAP treated group). At the same time, 6 mice were injected in the tail vein with 50  $\mu\text{L}$  of sterile 0.9% saline (DAAP-untreated group).

### Contrast-Enhanced Ultrasound Imaging

Microbubbles used for contrast-enhanced ultrasound (CEUS) were prepared according to our previously described method (17). Briefly, microbubble materials (DSPC, PEG 40 stearate, and DSPE-PEG2000-biotin) were weighed as dry solids and combined, followed by chloroform addition dropwise to the dry surfactant mixture until both components were completely dissolved. The solution was placed under a fume hood to allow evaporation until only a dry white film was left on the inside of the vial. Residual chloroform was then removed by placing the uncapped vial in a desiccator for 3 minutes. The film was hydrated with phosphate-buffered saline, an aqueous solution consisting of 121.5 mmol/L NaCl, 25.2 mmol/L  $\text{Na}_2\text{HPO}_4$ , and 4.8 mmol/L  $\text{KH}_2\text{PO}_4$ . After the addition of phosphate-buffered saline to the dried film with the phospholipid component, the vial was tightly capped and incubated at  $62^\circ\text{C}$  for a period of 2 to 4 hours to fully hydrate the coating materials.

Previous studies have shown strong correlations between histologic microvascular density and intensity of CEUS in the mouse tumor model (18–20). The modulation of RCC tumor microvasculature was evaluated and compared by CEUS as in previous studies. The CEUS evaluation was performed immediately before RF ablation with or without DAAP treatment in all mice.

To assess the contrast enhancement of the microbubbles, we used clinical ultrasound equipment (Vivid 7, GE Vingmed Ultrasound, Horten, Norway) in the grayscale pulse inversion mode. Ultrasound images were acquired with a broadband L9 transducer and a low mechanical index of 0.07. The images were recorded digitally and analyzed offline, and the video intensity within the tumor was measured using the ImageJ software (National Institutes of Health, Bethesda, MD, USA). The mice were placed in an induction chamber with 4% isoflurane in oxygen to induce anesthesia. During imaging, anesthesia of mice was maintained with 1.5% isoflurane in oxygen, and the mice were allowed to recover between image acquisitions. Ultrasound images of the tumor tissue were acquired at 1 minute after bolus intravenous injection of microbubbles. Each mouse was given a bolus injection of  $1 \times 10^6/\text{g}$  of microbubbles in 0.05

mL saline via the tail vein.

To assess the micro-vascular enhancement achieved with the contrast agent, the video intensity of the tumor tissue was determined and the contrast enhancement ratios (CER) were calculated in the regions of interest of the whole tumor volume by measuring both before and after microbubbles injection relative to the whole tumor volume. Quantitative analysis of each region of interest was used to extract the mean value of signal enhancement at each time point, and the CER were then calculated in each tumor tissue. The CER at the tumor tissue between precontrast (C0) and postcontrast (C1) images was calculated using the following equation:  $CER = (C1 - C0) / C0$ .

### RFA

Four mice treated with DAAP and 4 of the 6 mice in the DAAP-untreated group were subsequently treated with RFA 7 days after DAAP or sterile saline injection. The remaining 2 DAAP-untreated mice did not receive RFA therapy. As a result, this study included 3 groups: no-therapy (i.e., sterile saline injection only;  $n = 2$ ), RFA only (i.e., RFA following sterile saline injection;  $n = 4$ ), and RFA combined with DAAP treatment (i.e., RFA following DAAP injection;  $n = 4$ ). A 500-kHz RF generator (CC-1; Valley-Lab, Boulder, CO, USA) was used to apply conventional monopolar RF energy. This generator was selected because it is available for clinical use and is capable of monitoring impedance, tip temperature, and other parameters of the ablation. RF energy was applied for 5 minutes into the tumor, with the generator output titrated to maintain a designated tip temperature: a mean temperature of  $70 \pm 2^\circ\text{C}$  for each tumor. RF tip was performed using cool-tip type electrodes with a 1-cm active tip. RFA was performed as previously described by Horkan et al. (21) and Hines-Peralta et al. (22).

### Measurement of RF Ablation Effect

All mice were euthanized immediately after RFA with 4% isoflurane in oxygen. After the entire tumor was removed and embedded in agar, 3 slices were prepared using a vibratome. The area of coagulation necrosis was measured by virtual caliper and assessed mitochondrial enzyme

activity after incubation of the tumor samples in a 2% solution of 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) at  $37^\circ\text{C}$  for 30 minutes. The absence of mitochondrial enzyme activity accurately reflects irreversible cellular injury induced by percutaneous tumor ablation (23). With this assessment method of TTC staining, viable area with intact mitochondrial enzyme activity is stained red, while ablated area does not have a red color. The viable area of the tumor area was then calculated as a percentage of the viable area in the total area of the tumor and overlaid as a red color display on the photograph image using the image analysis tool (Matlab 7.4, Mathworks Inc., USA).

### Statistical Analysis

Modulation analysis of RCC tumor microvasculature was performed using the nonparametric Mann-Whitney U test. The TTC staining analysis for mitochondrial dehydrogenase activity was assessed with one-way analysis of variance (ANOVA) and post hoc Tukey's test. Data were presented as mean  $\pm$  standard deviation. Statistical significance was set at  $p < 0.05$ . All statistical analysis was performed using SPSS version 17.0 software (SPSS Inc., Chicago, IL, USA).

## RESULTS

### Tumor Measurement by Contrast-Enhanced Ultrasound Imaging

The mean tumor area measured at the largest cross-section by ultrasound before RFA was  $77.1 \pm 20.2 \text{ mm}^2$  and  $69.5 \pm 28.3 \text{ mm}^2$  in DAAP-untreated group and DAAP-treated group, respectively, at 7 days after administration DAAP, with no significant difference between groups (Table 1). CEUS images captured at 1 minute after intravenous injection of microbubbles showed lower signal intensity in DAAP-treated mice than in DAAP-untreated mice (Fig. 1). The CER for tumor vascularization on CEUS images were significantly lower in DAAP-treated group ( $30.2 \pm 9.9\%$ ) than in DAAP-untreated group ( $77.4 \pm 17.3\%$ ;  $p = 0.021$ ) (Fig. 2).

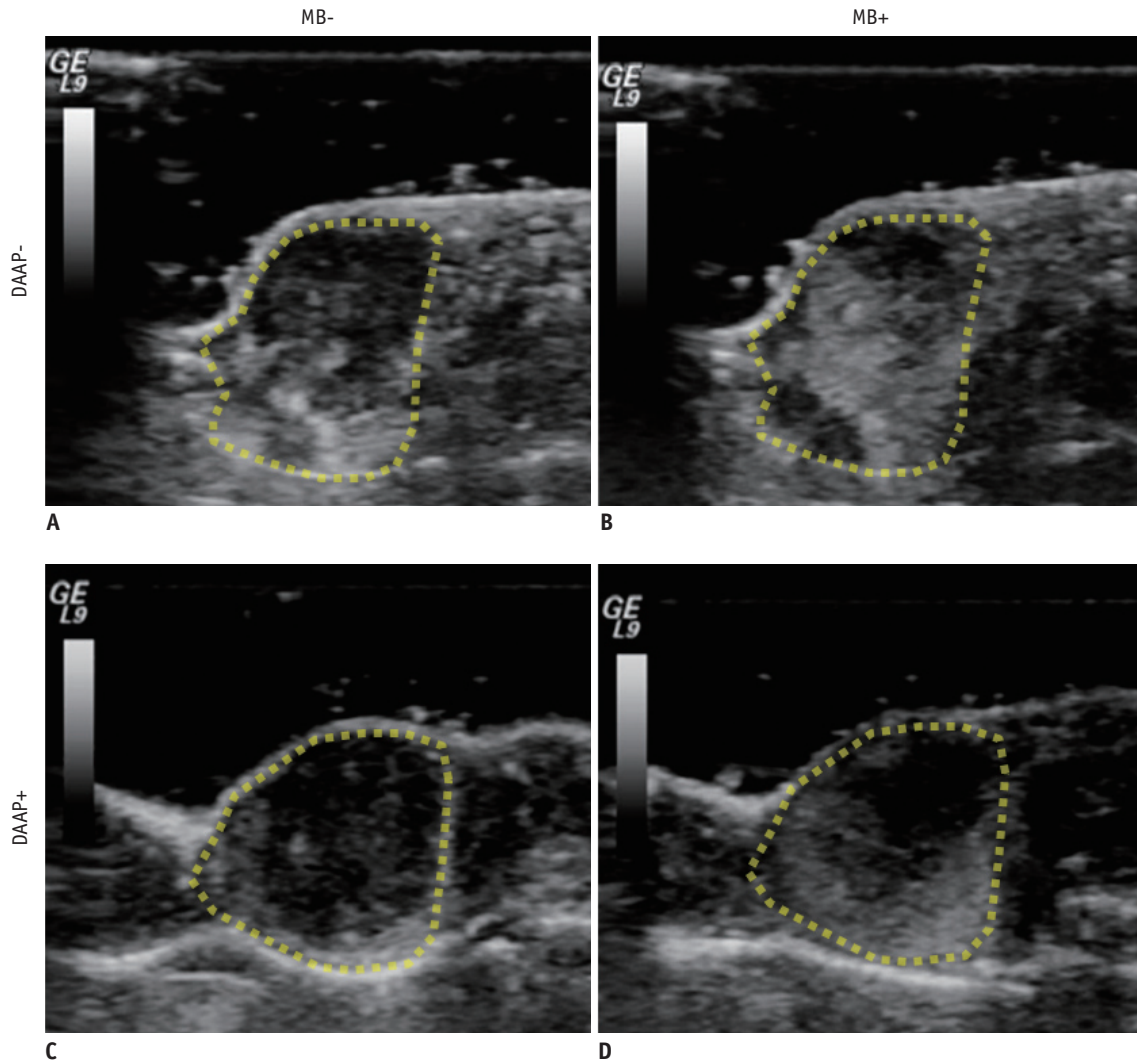
### Effect of DAAP on RF-Induced Tumor Coagulation

Figure 3 showed TTC staining in the 3 groups for

**Table 1. Tumor Measurement by Contrast-Enhanced Ultrasound Imaging**

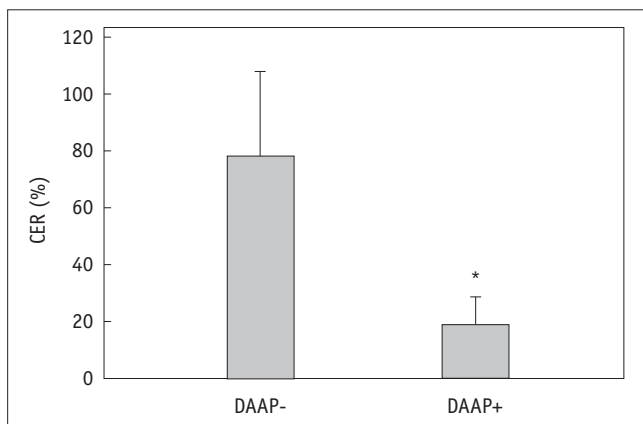
	DAAP-Untreated Group (n = 6)	DAAP-Treated Group (n = 4)	P
Tumor size (mm <sup>2</sup> )	77.1 $\pm$ 20.2	69.5 $\pm$ 28.3	0.517
Contrast enhancement ratio (%)	77.4 $\pm$ 17.3	30.2 $\pm$ 9.9	0.021

Statistical analysis was performed with nonparametric Mann-Whitney U test. DAAP = double anti-angiogenic protein



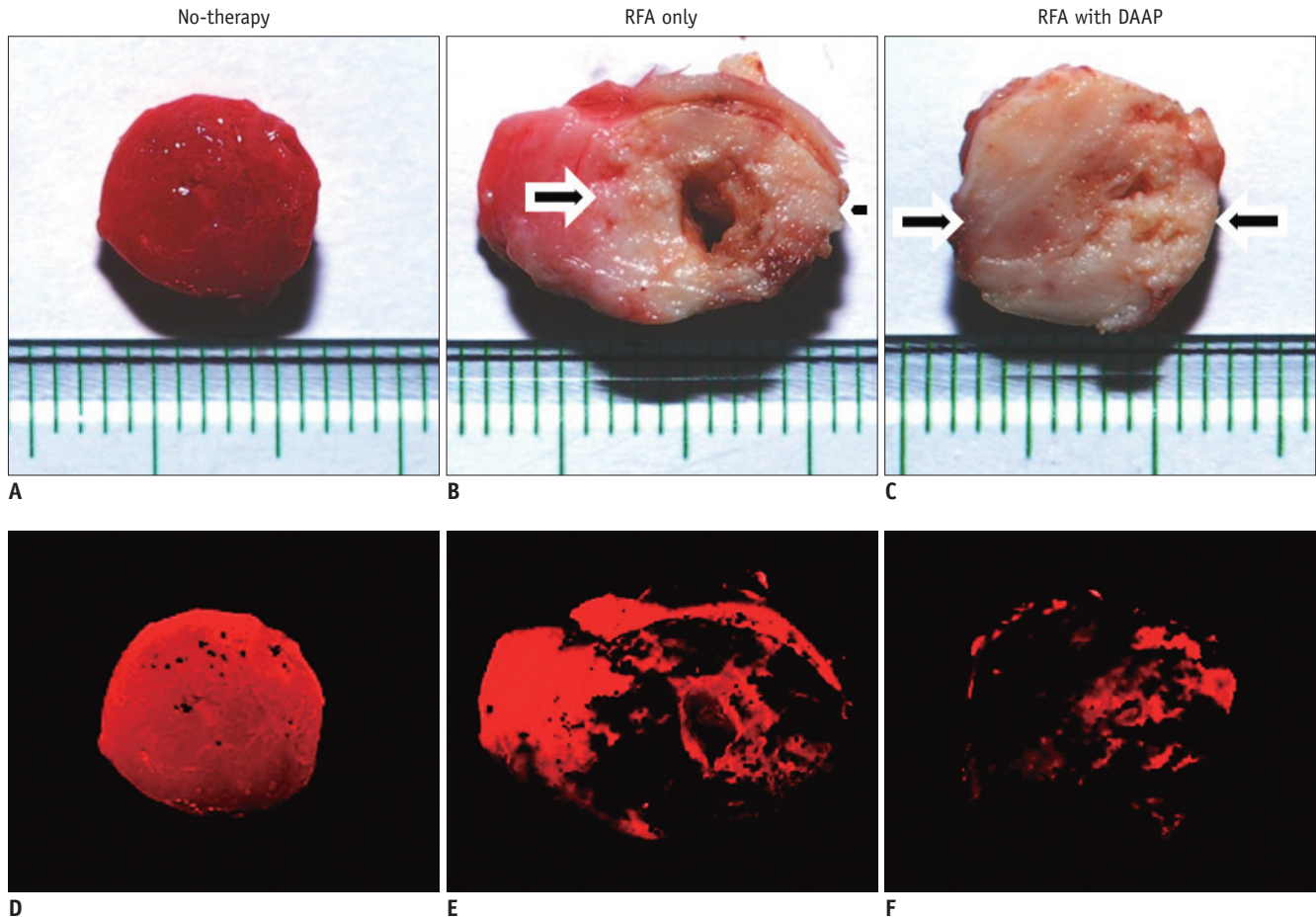
**Fig. 1. Comparison of contrast-enhanced ultrasound images of tumors after injection of microbubbles.**

**A, B.** Images of untreated tumor show strong enhancement in intratumoral area (yellow circle) after injection of microbubbles. **C, D.** In contrast, treated tumor with double anti-angiogenic protein (DAAP) shows similar contrast enhancement after injection of microbubbles.



**Fig. 2. Graph demonstrating contrast enhancement ratios (CER).** CER after injection of microbubbles was significantly different between untreated tumor and double anti-angiogenic protein (DAAP)-treated tumor. \* $p < 0.05$ .

assessment of the efficacy of RFA only and RFA combined with DAAP treatment. The mean coagulation diameter (white area) measured  $8.5 \pm 0.4$  mm in RFA combined with DAAP group,  $6.7 \pm 0.7$  mm in RFA only group, and 0 mm in no-therapy group (ANOVA,  $p < 0.001$ ) (Table 2). Using Matlab analysis, the area of viable mitochondria showed significant difference between the 3 groups (ANOVA,  $p < 0.001$ ). Figure 4 demonstrated the area of viable mitochondria within the tumor and its averaged values were summarized in Table 2. The area of viable mitochondria within the tumor was significantly smaller in RFA combined with DAAP group, as compared with RFA only group (red area,  $2.1 \pm 0.7\%$  vs.  $10.3 \pm 4.5\%$ ;  $p = 0.024$ ) and in RFA only group, as compared with no-therapy group (red area,  $10.3 \pm 4.5\%$  vs.  $27.9 \pm 3.9\%$ ;  $p = 0.001$ ).



**Fig. 3. Assessment for mitochondrial viable area.** Cross sections of three gross pathologic specimens stained with 2,3,5-triphenyl-2H-tetrazolium chloride. Mitochondrial activity is shown in red. Nonviable ablated tumor remains white (arrows). Mitochondrial activity is clearly seen in (A) untreated tumor but not in treated tumor with (B) radiofrequency ablation (RFA) only or with (C) RFA and double anti-angiogenic protein (DAAP). (D-F) Remaining viable mitochondria were overlaid as red color using MATLAB.

**Table 2. Effect of DAAP on RF-Induced Tumor Coagulation**

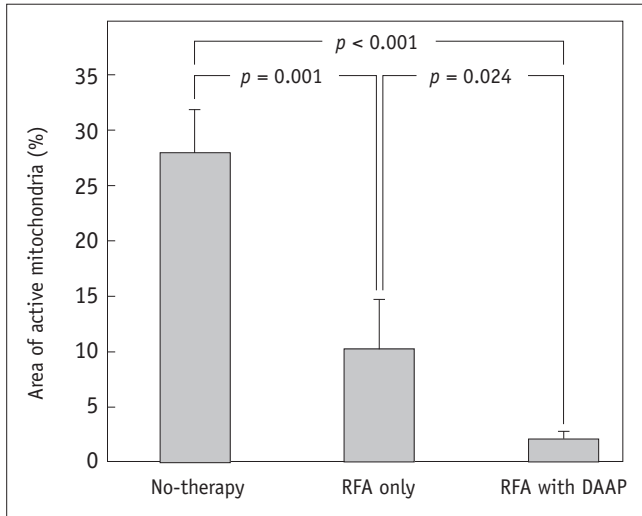
	No-Therapy (n = 2)	RFA Only (n = 4)	RFA with DAAP (n = 4)	P*
Coagulation diameter (mm)	0	6.7 ± 0.7	8.5 ± 0.4	< 0.001 <sup>†</sup>
Area of viable mitochondria (%)	27.9 ± 3.9	10.3 ± 4.5	2.1 ± 0.7	< 0.001 <sup>‡</sup>

\*Statistical analysis was performed with one-way ANOVA, <sup>†</sup>Post hoc test with Tukey's test: between 'no-therapy' and 'RFA only',  $p < 0.001$ ; between 'RFA only' and 'RFA with DAAP',  $p = 0.005$ ; between 'no-therapy' and 'RFA with DAAP',  $p < 0.001$ , <sup>‡</sup>Post hoc test with Tukey's test: between 'no-therapy' and 'RFA only',  $p = 0.001$ ; between 'RFA only' and 'RFA with DAAP',  $p = 0.024$ ; between 'no-therapy' and 'RFA with DAAP',  $p < 0.001$ . DAAP = double anti-angiogenic protein, RFA = radiofrequency ablation

## DISCUSSION

Our results suggested that pretreatment of RCC tumors with the antiangiogenic agent DAAP in nude mice can decrease tumor vascularity and substantially enhance coagulation when given in conjunction with RFA. This interaction is likely mediated by the well-documented antiangiogenic properties of DAAP. DAAP appears to distribute well in the tumor environment and block VEGF-A and Ang-2 in a synergistic manner, possibly via increasing

ligand aggregation because of increased avidity. DAAP has relatively high bioavailability and a longer half-life than either VEGF-Trap or Tie2-Fc. In addition, DAAP is effective in inducing tumor vessel regression and reducing subsequent blood flow in established tumors (14). The authors suggested that DAAP can be an effective therapeutic strategy for blocking tumor angiogenesis, metastasis, and vascular leakage. Regarding the toxicity, they reported that repeated DAAP treatment induced anticipated toxicities such as thrombocytosis, hypertension and microalbuminuria



**Fig. 4. Graph demonstrating area of viable mitochondria.** Tumor treated with RFA and DAAP showed significantly smaller zone of mitochondrial activity than no-therapy group and RFA-only group. *P* values among 'no-therapy', 'RFA only', and 'RFA with DAAP' are indicated in graph (one-way ANOVA followed by post hoc Tukey's test). ANOVA = analysis of variance, DAAP = double anti-angiogenic protein, RFA = radiofrequency ablation

for immune response (14).

Our study showed that the effect of DAAP could not be evaluated on gray-scale ultrasound. On the contrary, the CEUS images demonstrated that the CER for tumor vascularization were significantly lower in the DAAP treatment group, as compared to untreated group. To better understand the effect of DAAP, CEUS is a simple way to noninvasively image tumoral vascularity and detect any reductions in tumoral vascularity after treatment. CEUS is a widely used imaging technique in which microbubbles help to improve contrast between blood vessels and the surrounding tissue during ultrasound imaging. CEUS has been used to assess functional changes in response to antiangiogenic treatment in patients with RCC (23-25). Furthermore, it may enable more accurate prediction of RFA size and more precise determination of antiangiogenic therapy failure before RFA (26, 27). Direct assessment with CEUS may help to evaluate endpoints in combination therapy clinical trials.

Combination therapy of DAAP and RFA may help to overcome the size limitation. Specifically, reduction of the microvasculature density could cause cure in stage T1b tumors (> 4 cm of RCC) that cannot be treated effectively with RFA only (28). Furthermore, sufficient tumor destruction may be achieved with a reduction in the duration time of RFA therapy (29). Combination therapy could improve uniformity of heat deposition during RFA and

potentially reduce treatment failure.

We acknowledge several limitations of our study. First, our analysis was limited by the use of only 1 animal tumor model. Further investigation is needed to determine if the interaction observed here in RCC of nude mice can be generalized to other tumor types. As described above, DAAP has a broad spectrum of activity, but its activity when in combination with RFA may likely depend on the sensitivity of the tumor to antiangiogenic therapy, as well as on tumor size. Second, even though we used CEUS after DAAP therapy, we also measured CEUS at a single site in the tumor. Therefore, the antiangiogenic effects of DAAP in the global tumor remain unclear. For these reasons, a secondary method of vascularity analysis such as computed tomography or magnetic resonance perfusion imaging may enable more precise determination of the sufficiency of antiangiogenic therapy before RFA. Finally, treatment was provided for 9 days on the basis of observed changes in tumor size, but optimal dosing concentration and duration remain unclear and warrant further tumor-specific studies.

In conclusion, our results suggested the potential value of combining RFA with antiangiogenic therapy. However, further investigation is needed, including validation in other tumor types. In addition, combination of DAAP with other antiangiogenic therapies, as well as combinations with other forms of thermal ablation, such as microwave ablation, should be considered. Future studies should also include clinical trials of potentially additive therapies for the treatment of RCC and possibly other tumor types.

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