Profile of Serum Factors and Disseminated Tumor Cells Before and After Radiofrequency Ablation Compared to Resection of Colorectal Liver Metastases-A Pilot Study

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Abstract. *Background/Aim: The degree of systemic response after hepatic radiofrequency ablation (RFA) has not been well-compared to liver resection so far. This pilot study was designed to examine whether RFA, compared to liver resection, significantly varies concerning dissemination of circulating tumor cells and induction of different pro-inflammatory markers and liver-specific growth factors. Patients and Methods: Patients with colorectal liver metastases were treated with RFA, a combination of RFA and resection or liver resection only. Blood samples of 18 patients were obtained at different time points and interleukin (IL)-6, hepatocyte growth factor (HGF) and 70-kD heat shock protein (HSP70) serum levels were determined by ELISA. Circulating tumor cells were detected with reverse transcription-polymerase chain reaction (RT-PCR) amplification of cytokeratin 20 (CK20) mRNA (CK20 RT-PCR). Results: The detection of circulating tumor cells was not significantly different, but in two patients RFA induced tumor cell dissemination. Serum levels of IL-6 were strongly elevated after the operation without any significant differences between the treatment groups. The HGF ratio was significantly higher after RFA+resection compared to resection-alone and the HSP70 ratio also showed significantly higher values after RFA compared to resection alone. High postoperative IL-6 and HGF levels negatively influenced overall survival (OS) independently of the treatment group. Conclusion: This pilot study demonstrates that RFA might influence tumor cell dissemination. There exist detectable differences in serum factors between RFA and liver resection after the operation but this did not influence the overall survival of the patients. For all patients, high postoperative IL-6 and HGF levels are negative prognostic markers.*

New chemotherapeutic drugs have achieved decreased recurrence rates for patients with colorectal cancer (1), but metastases especially in the liver are major factors determining a worse prognosis for these patients. Hepatic resection still represents the gold standard for patients with colorectal liver metastases (CRLM). A five-year survival rate of 40% to 50%, depending on patient selection, was achieved in recent studies with complete surgical resection (2). Unfortunately, a curative resection is only possible in less than 25% of the patients at the time of diagnosis (3). The combination of effective neoadjuvant chemotherapy and strategies like portal vein embolisation and two-stage hepatectomy can increase the rate of secondary R0 resections of initially irresectable metastases up to 20% of the patients (4, 5).

In cases of non-resectable liver metastases, radiofrequency ablation (RFA) can be used as an alternative method. RFA is a local destructive technology that has been designed to destroy tumors in the liver. However, the exact role in the treatment of non-resectable liver metastases has still to be defined (6). Recently, the first randomized clinical trial evaluating the role of RFA *vs*. RFA plus chemotherapy in the treatment of CRLM was published (7). In this series, RFA plus chemotherapy resulted in significant longer progression free survival, but the effect of RFA on overall survival (OS) remains uncertain. There was a long debate regarding the use of RFA for resectable CRLM. A meta-analysis showed that resection of CRLM is significantly superior to RFA regarding OS (8).

Circulating tumor cells (CTC) reflect a minimal residual disease that cannot be eliminated by primary surgery. Tumor cell dissemination can already happen during early phases of tumor growth. In addition, it has been shown that tumor

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Table I. *Summary of the clinical characteristics of the patients.*

Factor	Category	RFA*	Resection
Recurrent LM**	Yes	3	2
	No	7	6
Age	Mean [years]	62	65
Number of LM	1	4	6
	2-5	5	2
	>5	1	0
Onset of LM	Synchronous	6	5
	Metachronous	4	3
Size of LM	<2 cm	0	0
	2-5 cm	6	5
	>5cm	4	3
Diameter of LM	Mean [cm]	5.5	5.3
Type of liver resection	Major	n.a.	4
	Minor	n.a.	4
Overall survival	Mean [months]	31	37
MSKCC***-Score	Mean [units]	3	3
Operating time	Mean [minutes]	273	312

*Radiofrequency ablation (RFA). **Liver metastases (LM). ***Memorial-Sloan-Kettering Cancer Center (MSKCC).

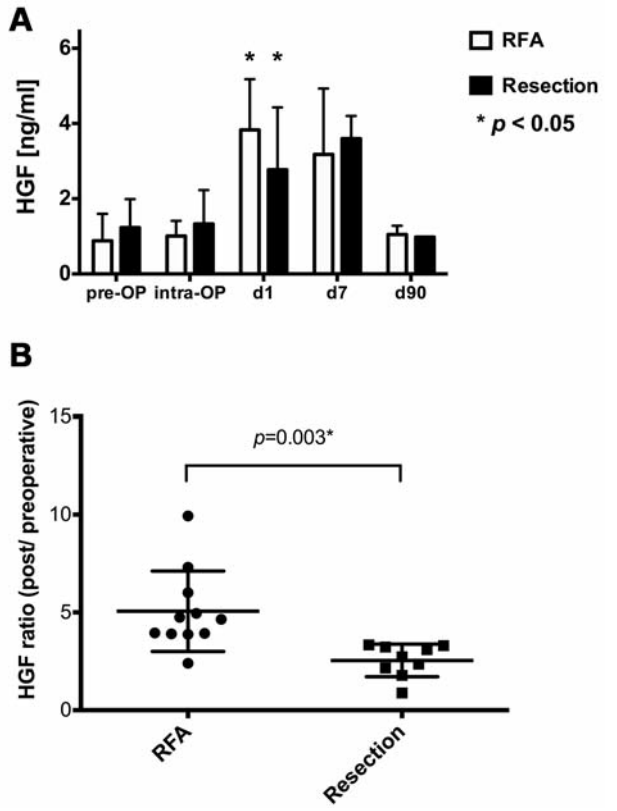

Figure 1. *A. Serum levels of hepatocyte growth factor (HGF) were measured by ELISA at different time points after treatment of liver metastases. Serum levels at d1 were significantly higher than before the operation (pre-OP). B. The ratio of HGF was calculated between serum levels at d1/ pre-OP.*

cell dissemination can be forced by intraoperative manipulation during liver resection (9). The risk of tumor cell dissemination during the application of RFA is apparent due to the high temperature, which occurs during the procedure. There have been reports demonstrating an intrahepatic tumor cell dissemination after RFA in hepatocellular carcinoma (HCC) patients (10). Only one study has so far evaluated the effect of RFA on intraoperative tumor cell dissemination in CRLM, showing no difference between surgical resection and RFA (11). We have previously shown that detection of CTC with reverse transcription-polymerase chain reaction (RT-PCR) amplification of cytokeratin 20 (*CK20*) mRNA in the bone marrow is a negative prognostic factor for patients after liver resection for colorectal liver metastases (12).

During the application of RFA, high temperatures are produced in the liver parenchyma. It was demonstrated that RFA induces up-regulation of soluble 70-kD heat shock protein (HSP70) expression (13). HSP70 has been shown to protect cells from many types of stress and apoptosis (14) and emerging evidence points out that HSP70 can stimulate the production of pro-inflammatory signals, such as Interleukin-6 (IL-6) and lead to a specific T-cell-mediated immune response against tumor cells (15). On the other hand, it has been shown that high levels of HSP70 are associated with worse prognosis in patients with colorectal cancer (16).

Liver regeneration after partial hepatectomy or liver damage is induced by an orchestration of different cytokines and growth factors. Activated non-parenchymal hepatic cells are involved and produce IL-6 (Kupffer cells) and hepatocyte growth factor (HGF) (stellate cells). These factors promote, together with other cytokines, hepatocyte entry through G_1 to the S phase of cell cycle, which leads to hepatocyte proliferation (17). IL-6 is indispensable during liver regeneration and absence of IL-6 results in liver failure and liver necrosis (18). On the contrary, several studies have shown that HGF and its receptor, c-Met, promote tumor cell invasion and tumor angiogenesis (19), which ultimately led to unfavorable prognosis of colorectal cancer patients with high HGF serum levels (20). Furthermore, for colorectal cancer patients, high IL-6 levels are associated with tumor progression, metastases and poor survival (21).

The aim of this pilot study was to evaluate if RFA, compared to liver metastasis resection, induces different amounts of intra-operative tumor cell dissemination. In addition, we investigated whether RFA and liver resection induce different serum levels of HGF, HSP70 and IL-6 and if time kinetics are different between the treatment modalities. Furthermore, the influence of the above mentioned serum factors and circulating tumor cells on OS were tested.

Table II. *Overview of the test results of circulating tumor cells with Cytokeratin 20 (CK20) RT-PCR before (pre-OP) during (intra-OP) and at day 1 (d1) after surgery.*

	Group 1 (RFA) (10 patients)			Group 2 (resection) (8 patients)		
Factor	Positive	Negative	No probe	Positive	Negative	No probe
Ck20 pre-OP	5	4	1	8	0	0
CK20 intra-OP	6	2	2	3	3	2
CK20 d1	6	4	0	4	3	1

Patients and Methods

Patients. In this pilot study, patients with colorectal liver metastases who underwent surgery at the Department of General and Thoracic Surgery, University Hospital Kiel, Germany, have been included. Informed consent was obtained from all patients prior to enrollment in the study. The local ethics committee of the University Kiel approved the study (AZ: A123/01) and all procedures followed the Declaration of Helsinki concerning ethical standards. The extent of the metastases was assessed with computed tomography (CT) or magnetic resonance imaging (MRI) prior to surgery. Intraoperative assessment of the resectability of the metastases and the determination of the intraoperative treatment strategy was assessed by routinely performed intraoperative ultrasound. The decision of performing RFA or resection was dependent on the local tumor situation, *e.g*. bilobar disease, vessel invasion and remaining liver tissue. Patient data was prospectively documented in our oncology data bank (PROWebDB). Patients were followed-up and clinical course was documented. Statistical analyses were performed with the statistical package SPSS (version 22.0) and GraphPad Prism 6 (GraphPad, La Jolla, CA, USA).

Blood samples, RT-PCR and ELISA. Blood samples (10ml) were obtained from each patient at different time points: Before skin incision (pre-OP), during the operation before skin closure (intra-OP), 24h after the operation (d1), at day seven (d7) and after 12 weeks (d90). RNA was extracted from blood mononuclear cells (MNC) using density centrifugation through Ficoll-Hypaque. As described previously, RNA was extracted from MNC fractions and CK20-RT-PCR was performed (22). Samples were tested twice; in the case of two inconsistent results a third analysis was performed. Serum HGF and IL-6 concentrations were tested using human HGF and IL-6 ELISA (R&D Systems, Abingdon, UK) and HSP70 concentration was determined using HSP-70 high sensitivity ELISA (Assay designs, Ann Arbor, Michigan, USA) following the manufacturer's instructions.

Results

Patients' characteristics and follow-up. Eighteen patients were enrolled in the study and were operated within a period of 12 months. Eleven patients suffered from colon cancer, 7 patients had a history of rectal cancer. The median age was 66 years (range=48-78). The study cohort consisted of 11 males and 7 females. Five patients had received previous surgery for liver metastases. Ten of the patients had a history of former chemotherapy treatment. None of the patients was treated with neoadjuvant chemotherapy within three months before the operation. In three patients, bile leaks occurred after liver resection/RFA, treated with percutaneous drainage, and, in one patient, a pulmonary embolism due to a deep vein thrombosis was diagnosed. The median OS of all patients was 32 months. Sixteen patients died during follow-up due to tumor recurrence, two patients are still alive, one of them without any signs of tumor recurrence. Patients were divided into two groups: In group I, only RFA was used for liver metastases destruction (10 patients), in group II, resection of liver metastases was performed (8 patients). In two patients of group I, RFA was combined with small atypical liver resections. Table I summarizes the clinical characteristics of all patients in the two groups that include

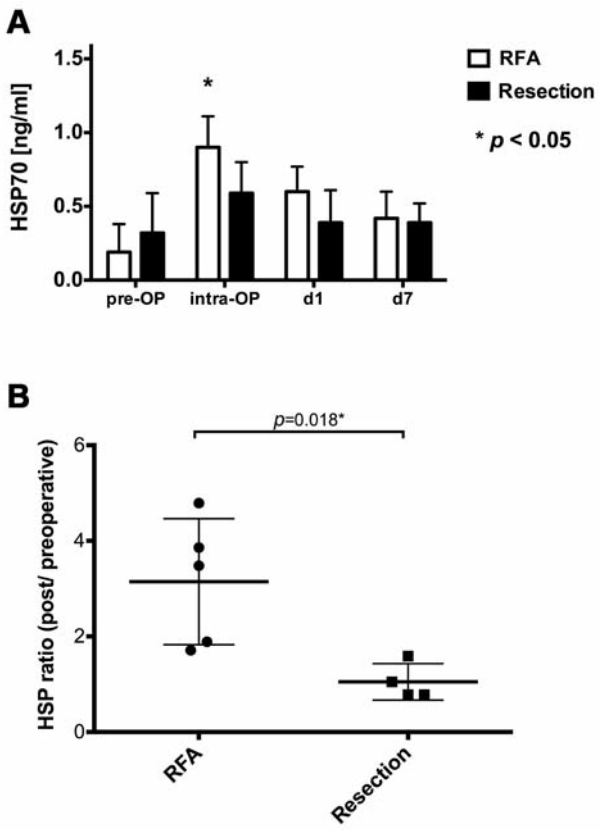

Figure 2. *A. Serum levels of heat shock protein 70 (HSP70) were measured by ELISA at different time points after treatment of liver metastases. Serum levels after RFA were significantly higher during the operation (intra-OP) compared to pre-OP levels. B. The ratio of HSP70 was calculated between serum levels at d1/ pre-OP.*

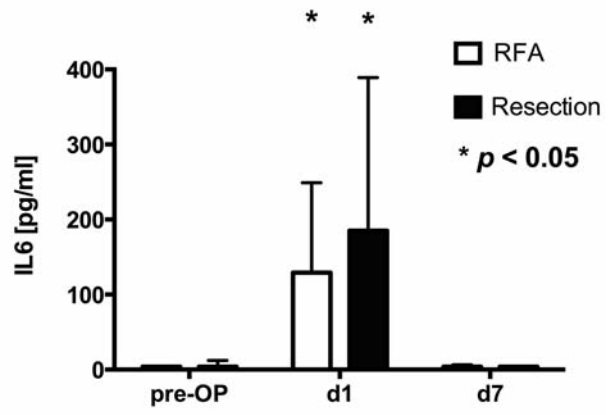

Figure 3. *Serum levels of Interleukin-6 (IL-6), as determined by ELISA at different time points after treatment of liver metastases.*

number and size of liver metastases, as well as type of liver resection, including the Memorial-Sloan-Kettering Cancer Center Score (MSKCC-Score) (23).

Analysis of circulating tumor cells with CK20 RT-PCR. The overall detection rate of CTC in the blood of all patients prior to operation was 76 % (13/17). In group I (RFA), the detection rate was 56 % (5/9) before the operation. In all patients of group II (resection), CTC were detectable prior to operation (8/8). Directly after the operation (d1), CTC were detected in 59 % of the patients. In two patients of group I with initial negative detection of CTC before RFA, CTC were detectable after the operation. Conversely, in group II, CTC were detected in all patients prior to resection of liver metastases and in three patients, CTC were not detectable any more after the operation (Table II).

Serum concentrations of HGF, HSP-70 and IL-6. The serum levels of HGF were detected before, during and after the operative procedure. In all patients, the operative procedure led to an induction of HGF serum levels (Figure 1A). There was a significant increase of HGF serum levels after resection of liver metastases and RFA compared to preoperative values ($p<0.05$; *t*-test, two-tailed). In our time course of HGF levels (pre-OP, intra-OP, postoperative day 1, day 7 and day 90), we measured the serum peak level after liver resection at postoperative day 7. After RFA, the postoperative peak was reached at day 1. Ninety days after the operation, HGF levels were not elevated in any patient. To determine the influence of the operative procedure on HGF serum levels, we determined the ratio of post- (d1) and pre-operative serum levels. Analysis showed that the use of RFA resulted in a significantly higher HGF ratio than resection of liver metastases ($p=0.003$, *t*-test, two-tailed) (Figure 1B).

We further evaluated to which extent the operative procedure influenced the release of HSP70 in the serum. HSP70 levels increased during the operative procedure reflecting a shorter time course for protein induction compared to HGF. RFA led to a significant increase compared to preoperative HSP70 levels ($p<0.05$; *t*-test, two tailed) (Figure 2A). Resection of liver metastases induced only a non-significant increase of HSP70 levels. The HSP70 ratio (post-/ preoperative serum levels) showed significant higher serum levels after RFA compared to resection alone ($p=0.018$, *t*-test, two-tailed) (Figure 2B).

Serum levels of IL-6 were not elevated above the normal value in any patient prior to the operation. After RFA, the mean level of IL-6 in the serum was 129 pg/ml and 185 pg/ml after liver resection. The serum levels of IL-6 were significantly higher after the operative procedure at day 1 ($p<0.05$; *t*-test, two-tailed) but not significantly different between the two treatment groups (Figure 3). Seven days after the operation, the IL-6 serum levels had already reached preoperative levels.

Correlation of serum markers and CTC with OS. The median OS time of all patients was 22 months. OS was not significantly different between the two treatment groups. High postoperative HGF levels (above 4 ng/ml) were strongly associated with subsequent worse OS (Figure 4). The median OS for patients with low HGF after the operation was 25 months, whereas the OS for patients with high HGF was 19 months ($p=0.024$; log-rank test). The pre- or postoperative detection of CTC in the blood did not significantly influence OS in this group of patients. OS was also independent of HSP-70 levels before or after the operation (data not shown).

Four patients with very high postoperative IL-6 levels (>200 pg/ml) also showed a significant worse OS. The OS in these patients, was 17 months, whereas in patients with low IL-6 (<200 pg/ml), OS was 25 months ($p=0.05$). We found no correlation between tumor size, number of metastases, extend of liver resection or treatment modality (RFA *vs.* resection) in patients with very high postoperative IL-6.

Discussion

RFA is a local treatment option for different tumors in the liver, including metastases, HCC and other primary tumors of the liver as we have described before (24). The use of RFA in the treatment of resectable CRLM is now being questioned. A recent meta-analysis showed that patients treated with RFA have an inferior prognosis compared to patients after resection of the metastases (8). In contrast, there have been several reports describing anti-tumor effects and systemic effects beyond local tumor control induced after RFA (25). For this reason, in the present pilot study, we analyzed several serum factors, including tumor cell dissemination after RFA compared to resection of CRLM to better understand the systemic effects of local RFA treatment.

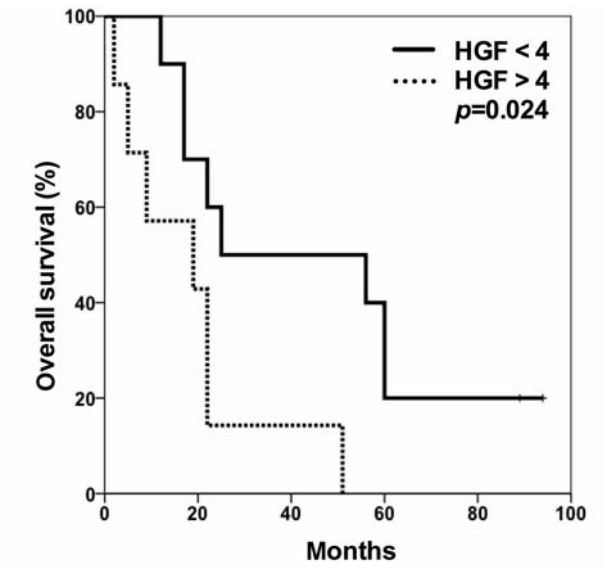

Figure 4. *Kaplan-Meier analysis of overall survival in all patients stratified by high or low HGF serum levels [ng/ml] at day 1 after the operative procedure.*

Tumor cell dissemination is a precondition for the development of distant metastases. According, however, to the revisited "seed and soil" hypothesis, local environmental factors are also important for the formation of metastases (26). Detection of circulating tumor cells / disseminated tumor cells (CTC/ DTC) in blood and bone marrow after liver metastases resection are negative prognostic factors (12, 27). To which extent the resection technique of CRLM or the use of RFA influences intraoperative tumor cell dissemination is uncertain and rarely studied. A prospective randomized trial comparing two different liver resection techniques concerning the extent of tumor cell dissemination has not yet been published (28). Topal *et al*. have been the first to quantitatively compare hematogenous cancer cell dissemination after resection or RFA of CRLM, revealing no differences between these techniques relating to tumor cell spread in the blood (11). In three subjects of our series of patients, we observed that CTC were not detectable any more after resection of CRLM. On the other hand, two patients shifted from initial negative detection status to positive detection during and after RFA. This might implicate that RFA promotes tumor cell dissemination. However, larger patient series are required to thoroughly evaluate the influence of the resection technique of CRLM and its influence on intraoperative cancer cell dissemination. Furthermore, the influence of intraoperative tumor cell dissemination on tumor recurrence and survival has to be elucidated as it has been shown that most of the cells of an intraoperative tumor cell shedding do not survive and are already cleared after 24 h (29).

Surgical procedures themselves induce a variety of cytokines, which promote a general inflammatory reaction. IL-6 is a pro-inflammatory cytokine that has, besides its role in liver regeneration, a crucial role in tumor progression and modulation of immune function. It has been shown that IL-6 is important for the local inflammatory response in colorectal cancer by affecting T-regulatory cells (Treg) and also for the trafficking and recruitment of immune cells (30). We have measured serum IL-6 levels at different time points before and after liver resection or RFA, observing a strong release of IL-6 in the serum after the operative procedure. Resection of liver metastases led to the highest increase of IL-6 levels and, although not significantly higher than after RFA, it might implicate that resection reflects a higher "surgical trauma" to the liver than RFA. There was a significant correlation between IL-6 serum levels and OS. In a group of patients with high IL-6 levels at day 1 after the operation, OS was significantly worse than in the group with low IL-6 levels. For colorectal cancer, there have been several reports showing association of high serum IL-6 levels and unfavorable prognosis (21, 31). Besides the positive function of IL-6 on local tumor environment and its indispensable role in liver regeneration, very high serum IL-6 levels seem to be a negative prognostic factor for patients after liver metastases resection or RFA.

An association between serum HGF level and prognosis in malignant disease has been reported for patients with colorectal cancer (20). The activated HGF-related c-Met pathway is indispensable for liver regeneration after major liver resection, which is also a critical inducer for colorectal cancer disease progression. There was concern that major liver resection may induce tumor growth in the residual liver parenchyma or in distant organs. It has been reported that RFA promotes proliferation of residual intrahepatic neoplastic cells in an *in vivo* model compared to liver resection (32). Other groups described that hepatic resection, but not RFA, results in tumor growth and increased HGF expression *in vivo* (33). In our set of patients, we observed a strong induction of serum HGF after RFA or resection without a significant difference between the treatment groups; however, we observed a higher HGF ratio (post/ preoperative level) in patients treated with RFA. Interestingly, the peak of HGF levels were already reached at day 1 after RFA and prolonged to day 7 after resection of the metastases. An explanation may be that after resection the delayed elevation of HGF resembles the process of liver regeneration, whereas, after RFA, HGF elevation resembles the effect of liver tissue destruction, which is already apparent directly after the operation. High postoperative HGF levels were a significant prognostic marker decreasing OS in our patients as previously reported (34).

During RFA, high temperatures are induced in the liver due to the application of alternating current. Temperatures

above 60˚C induce cell death by coagulative necrosis. HSP70 expression is induced in the tumor cells shortly after RFA and systemically released into the circulation, most probably related to coagulative necrosis. We observed a significant elevated HSP70 ratio after RFA, but not after resection alone. Liver resection in this series of patients was performed with either the clamp-crush technique or with the use of a Cavitron Ultrasonic Surgical Aspirator (CUSA). There was no correlation between the extent of the coagulation (as estimated by the size of the treated metastases) and serum HSP70 levels, which is in accordance with other reports (13). Despite the proposed role of HSP70 in the initiation of an anti-tumor immunity, we were not able to measure differences in the OS of patients with a high HSP70 ratio compared to patients with low HSP70 ratio.

One limitation of this study is the small sample size in the two treatment groups regarding statistical analysis. Nevertheless, this pilot study to elucidate the systemic impact of RFA is the first to analyze different serum factors and circulating tumor cells before and after RFA and liver resection. In summary, we examined a subset of three different serum factors and CTC in patients with colorectal liver metastases, who were treated either with liver resection or RFA. Serum levels of IL-6 were strongly elevated after the operation without any significant differences between the treatment groups. The HGF ratio was significantly higher after RFA compared to resection alone and the HSP70 ratio also showed significantly higher values after RFA compared to resection alone. The detection of CTC was not significantly different between the treatment groups in this small series of patients; however, we observed two patients with increased numbers of CTC during and after RFA, which might implicate that the risk of tumor cell dissemination during RFA is an apparent problem. High postoperative IL-6 levels, equivalent to a strong systemic inflammatory reaction, negatively influenced OS independently of the treatment group. Whether this influence is only due to systemic reactions or also due to local impact of IL-6 on hepatocytes, remains elusive. Furthermore, high HGF levels also correlated with negative OS independently of the treatment group. It is noteworthy that the OS of the patients was not influenced by the detectable differences in serum factors between RFA and liver resection. Therefore, to evaluate the systemic effects of RFA and their prognostic impact, further studies with larger patient series are needed.

Conflicts of Interest

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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