

Treatment of advanced hepatocellular carcinoma with very low levels of amplitude-modulated electromagnetic fields

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BACKGROUND: Therapeutic options for patients with advanced hepatocellular carcinoma (HCC) are limited. There is emerging evidence that the growth of cancer cells may be altered by very low levels of electromagnetic fields modulated at specific frequencies.

METHODS: A single-group, open-label, phase I/II study was performed to assess the safety and effectiveness of the intrabuccal administration of very low levels of electromagnetic fields amplitude modulated at HCC-specific frequencies in 41 patients with advanced HCC and limited therapeutic options. Three-daily 60-min outpatient treatments were administered until disease progression or death. Imaging studies were performed every 8 weeks. The primary efficacy end point was progression-free survival ≥ 6 months. Secondary efficacy end points were progression-free survival and overall survival.

RESULTS: Treatment was well tolerated and there were no NCI grade 2, 3 or 4 toxicities. In all, 14 patients (34.1%) had stable disease for more than 6 months. Median progression-free survival was 4.4 months (95% CI 2.1–5.3) and median overall survival was 6.7 months (95% CI 3.0–10.2). There were three partial and one near complete responses.

CONCLUSION: Treatment with intrabuccally administered amplitude-modulated electromagnetic fields is safe, well tolerated, and shows evidence of antitumour effects in patients with advanced HCC.

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Treatment of inoperable or metastatic solid tumours is a major challenge in oncology, which is limited by the small number of therapeutic agents that are both well tolerated and capable of long-term control of tumour growth. Hepatocellular carcinoma (HCC) is the second most common cause of cancer death in men and the sixth in women worldwide (Jemal *et al*, 2011). Hepatocellular carcinoma is the most common tumour in certain parts of the world, particularly in East Asia, Africa, and certain countries of South America. This tumour is less frequent in Europe and in the United States, but has become the fastest rising cancer in the United States (Jemal *et al*, 2011). In the United States alone, it is estimated that 24 120 new cases were diagnosed and there were 17 430 deaths from HCC in 2010 (Jemal *et al*, 2010), a 27% increase in the number of new cases since 2004 (Jemal *et al*, 2004). The

prognosis of patients suffering from advanced HCC is poor with an average survival of fewer than 6 months (Kassianides and Kew, 1987; Jemal *et al*, 2011).

Therapies for HCC are limited. Resections of the primary tumour or liver transplantation are the preferred therapeutic approaches in patients who are surgical candidates (Bruix and Sherman, 2005). Although these interventions result in long-term survival for some patients, only a minority benefit from them because of limitations due to tumour size, patient's overall condition, and presence of hepatic cirrhosis (Cance *et al*, 2000). Only a small number of randomised trials show a survival benefit in the treatment of HCC. Chemoembolisation has been shown to confer a survival benefit in selected patients with unresectable HCC (Llovet *et al*, 2002). Data from two phase III randomised placebo-controlled studies demonstrate improved survival in patients with advanced HCC receiving the multikinase inhibitor sorafenib (Llovet *et al*, 2008b; Cheng *et al*, 2009). Additional therapies for this disease are sorely needed, especially for the large number of patients with advanced disease who cannot tolerate

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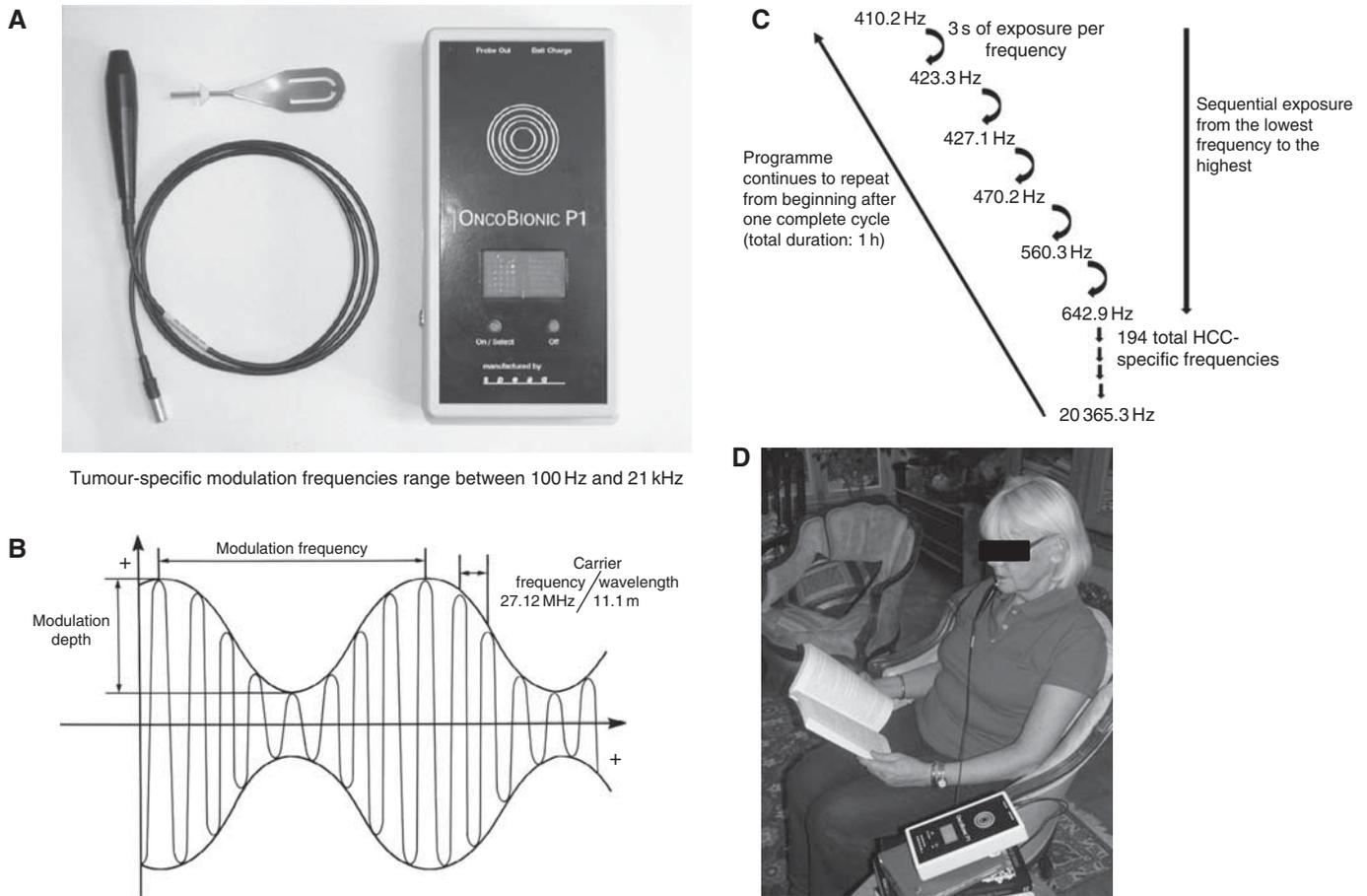


Figure 1 Delivery of HCC-specific modulation frequencies. **(A)** The generator of AM EMFs is a battery-driven RF EMF generator connected to a spoon-shaped mouthpiece. **(B)** Schematic description of AM EMFs. The carrier frequency (27.12 MHz) is sinusoidally modulated at specific frequencies. **(C)** Patient receiving treatment with RF AM EMF. **(D)** HCC treatment programme consisting of sequential emission of 194 modulation frequencies for 60 min.

chemotherapy or intrahepatic interventions because of impaired liver function (Thomas and Zhu, 2005).

The intrabuccal administration of low and safe levels of electromagnetic fields, which are amplitude-modulated at disease-specific frequencies (RF AM EMF) (Figure 1), was originally developed for the treatment of insomnia (Pasche *et al*, 1990). The highest levels of EMFs encountered during treatment are found at the interface between the tongue and the mouth probe and are compliant with international safety limits (ICNIRP, 1998; Pasche and Barbault, 2003). Tumour-specific modulation frequencies have been identified for several common forms of cancer and one report suggests that this novel therapeutic approach is well tolerated and may be effective in patients with a diagnosis of cancer (Barbault *et al*, 2009). However, the safety and potential efficacy of this treatment approach in the treatment of advanced HCC are unknown. We designed this single-group, open-label, phase I/II study to assess the feasibility of this treatment in patients with advanced HCC and limited therapeutic options.

PATIENTS AND METHODS

Patients

The study was aimed at offering treatment to patients with Child–Pugh A or B advanced HCC and limited therapeutic options. Patients were classified as having advanced disease if they were not

eligible for surgical resection or had disease progression after surgical or locoregional therapies or had disease progression after chemotherapy or sorafenib therapy. Patients with measurable, inoperable HCC were eligible for enrolment. Previous local or systemic treatments were allowed as long as they were discontinued at least 4 weeks before enrolment. Inclusion criteria included Eastern Cooperative Oncology Group performance status of 0, 1, or 2 and biopsy-confirmed HCC. Also allowed were patients with no pathological confirmation of HCC with a level of α -fetoprotein higher than 400 ng ml^{-1} and characteristic imaging findings as assessed by multislice computer tomography (CT) scan or intravenous contrast ultrasound (US). As per the University of São Paulo Department of Transplantation and Liver Surgery guidelines, liver biopsies are avoided in patients eligible for transplant or with severely impaired liver function. Exclusion criteria included confirmed or suspected brain metastasis, Child–Pugh C, previous liver transplant, and pregnancy.

Study design

This was an investigator-initiated, single centre, uncontrolled phase I/II trial in patients with advanced HCC. The trial was approved by the local human investigation committee and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from each patient. The protocol was registered: clinicaltrial.gov identifier no. NCT00534664.

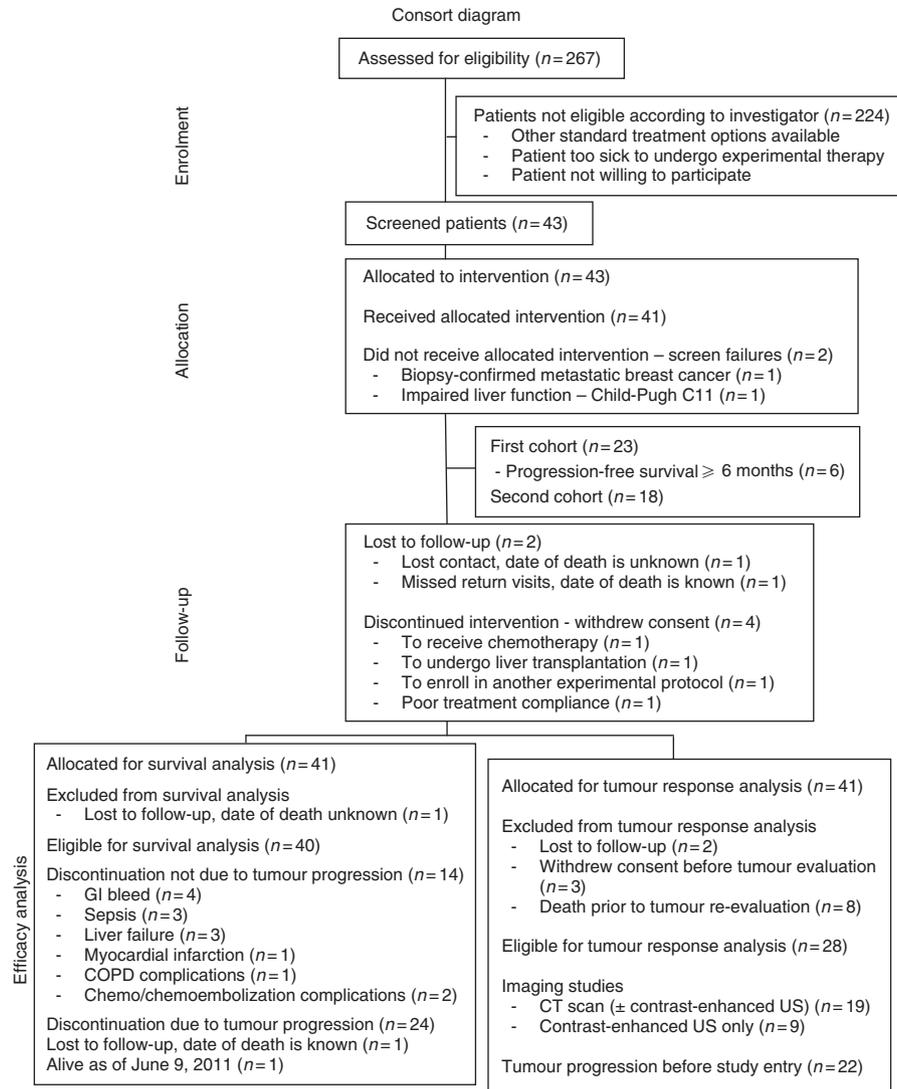


Figure 2 CONSORT diagram.

Administration of AM EMFs

The generator of AM EMFs consists of a battery-driven radio-frequency (RF) EMF generator connected to a 1.5 m long 50 Ω coaxial cable, to the other end of which a stainless-steel spoon-shaped mouthpiece is connected via an impedance transformer (Figure 1A). The RF source of the device corresponds to a class C amplifier operating at 27.12 MHz. The carrier frequency is AM (Figure 1B) with a modulation depth of $85 \pm 5\%$, whereas the modulation frequency is generated by a digital direct synthesiser with a resolution of 10^{-7} . The treatment sequence is controlled by a microcontroller (Atmel AT89S8252, Fribourg, Switzerland), that is, duration of session, sequence of modulation frequencies and duration of each sequence can be programmed via PC over a RS232 interface. The RF output is adjusted to 100 mW into a 50 Ω load, which results in an emitting power identical to that of the device used for the treatment of insomnia (Pasche *et al*, 1990; Reite *et al*, 1994; Pasche *et al*, 1996). The United States Food and Drug Administration has determined that such a device is not a significant risk device and it has been used in several studies conducted in the United States (Reite *et al*, 1994; Pasche *et al*, 1996; Kelly *et al*, 1997). A long-term follow-up survey of 807 patients who have received this therapy in the United States, Europe and

Asia showed that the rate of adverse reactions was low and was not associated with increases in the incidence of malignancy or coronary heart disease (Amato and Pasche, 1993). The maximum specific absorption rate (SAR) of the applied RF averaged over any 10 g of tissue has been estimated to be less than 2 W kg^{-1} , and the maximum temperature increase is significantly lower than 1°C anywhere in the body owing to RF absorption. The induced RF field values within the primary and metastatic tumours are significantly lower. In contrast, the RF fields induced during RF ablation of tumours cause hyperthermia and result in SAR in the range of $2.4 \times 10^5 \text{ W kg}^{-1}$ (Chang, 2003), that is, more than 100 000 times higher than those delivered by the device used in this study.

We have previously reported the discovery of HCC-specific modulation frequencies in 46 patients with HCC using a patient-based biofeedback approach and shown the feasibility of using AM EMFs for the treatment of patients with cancer (Barbault *et al*, 2009). The treatment programme used in this study consisted of three-daily outpatient treatments of 1 h duration, which contained HCC-specific modulation frequencies ranging between 100 Hz and 21 kHz administered sequentially, each for 3 s (Figure 1C and Supplementary Table S1).

The treatment method consists of the administration of AM EMFs by means of an electrically conducting mouthpiece, which is

in direct contact with the oral mucosa (Figure 1D). The patients were instructed on the use of the device and received the first treatment at the medical centre's outpatient clinic. A device was provided to each patient for the duration of the study. The patients were advised to self-administer treatment three times a day. Treatment was administered until tumour progression was objectively documented. At that time, treatment was discontinued. Treatment compliance was assessed at every return visit by recording the number of treatments delivered in the preceding 2 months.

Efficacy end points and disease assessment

The primary end point of this trial was the proportion of patients progression-free at 6 months. Secondary end points were progression-free survival (PFS) (first day of treatment until progression of disease or death) and overall survival (OS) (first day of receiving treatment to death). Objective response was assessed using the Response Evaluation Criteria in Solid Tumours group classification for patients with disease assessed by either helical multiphase CT (Therasse *et al*, 2000). Whenever contrast-enhanced US radiological assessment was used, it was performed and reviewed by the same radiologist specialised in HCC (MCC) as this imaging modality is investigator dependent. Tumour measurements were performed at baseline and every 8 weeks. Only patients with at least one repeat tumour measurement during therapy were considered for response analysis. Throughout the study, lesions measured at baseline were evaluated using the same technique (CT or contrast-enhanced US). Overall tumour response was scored as a complete response (CR), partial response (PR), or stable disease (SD) if the response was confirmed at least 4 weeks later. Alpha-fetoprotein (AFP) levels were measured every 8 weeks in all patients throughout the study, but changes in AFP were not an end point for assessment of response. Pain was assessed according to the NCI-CTCAE v.3.0 (http://ctep.cancer.gov/protocolDevelopment/electronic_applications/docs/ctcae3.pdf).

Statistical analyses and efficacy assessment

All eligible patients who began treatment were considered assessable for the primary and secondary end points. A Simon two-stage phase II minimax design was used (Simon, 1989) to evaluate the rate of progression-free survival at 6 months. The interim analysis was performed once enrolment into the first stage was completed. In the first stage, 23 patients were observed. If two or fewer patients had progression-free survival ≥ 6 months, the trial would be terminated early for lack of efficacy. If the progression-free survival of 3 or more of the first 23 patients was equal or greater than 6 months, then an additional 18 patients would be enrolled to a maximum of 41 patients. If eight or more of the 41 had PFS of at least 6 months, we would conclude that the treatment was efficacious. This design had a Type I error rate of 5% and a Type II error rate of 10% for the null hypothesis of a 6-month PFS rate of 10% vs the alternative of 27.5%. Kaplan–Meier estimates of survival, PFS, and duration of response were calculated with standard errors based on Greenwood's formula. These calculations were performed using the Proc Lifetest in SAS 9.2 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Patient recruitment and follow-up

From October 2005 to July 2007, 267 patients were assessed for eligibility (Figure 2). In all, 43 patients with advanced HCC and Child–Pugh A or B were enrolled in this study. The date of last patient follow-up is 9 June 2011. Of these, 20 patients (46.5%) had histological confirmation of HCC; 23 patients (53.5%) were

Table 1 Treatments received by patients with advanced HCC before enrolment (n = 41)

No previous treatment	7
Chemoembolisation	25
¹³¹ I-Lipiodol	1
Octreotide	1
Percutaneous alcohol injection therapy	1
Surgery	9
Systemic chemotherapy or sorafenib	5

Abbreviation: HCC = hepatocellular carcinoma. Two patients had surgery and chemoembolisation, two patients had surgery and systemic chemotherapy, one patient had surgery and chemoembolisation and systemic chemotherapy, one patient had surgery and percutaneous alcohol injection, one patient had surgery and sorafenib, one patient had chemoembolisation and systemic chemotherapy and one patient had surgery and octreotide.

Table 2 Patients' baseline characteristics

	No.	%
Age (years)		
Median age	64	
Range	18–85	
≥ 65	19	46.3
< 65	22	53.6
Sex		
Female	6	14.6
Male	35	85.4
ECOG performance status		
0	5	12.2
1	28	68.3
2	8	19.5
Child–Pugh status		
A5	15	36.6
A6	2	4.9
B7	6	14.6
B8	5	12.2
B9	11	26.8
No cirrhosis	2	4.9
BCLC status		
B	6	14.6
C	35	85.4
AFP > ULN		
Yes	28	68.3
No	13	16.7
Aetiology		
ETOH	2	4.9
Hepatitis B	6	14.6
Hepatitis B+C	1	2.4
Hepatitis C	22	53.7
ETOH+hepatitis C	1	2.4
NOS	9	22.0
Portal thrombosis	10	24.3
Extrahepatic disease		
Yes	16	39.0
Location		
Lung	6	14.6
Bone	3	7.3
Lymph nodes	4	9.8
Peritoneal carcinomatosis	1	2.4
Adrenal gland	1	2.4

Abbreviations: AFP = α -fetoprotein; BCLC = Barcelona Clinic Liver Cancer; ECOG = Eastern Cooperative Oncology Group; ETOH, ethyl alcohol; ULN, upper limit of normal.

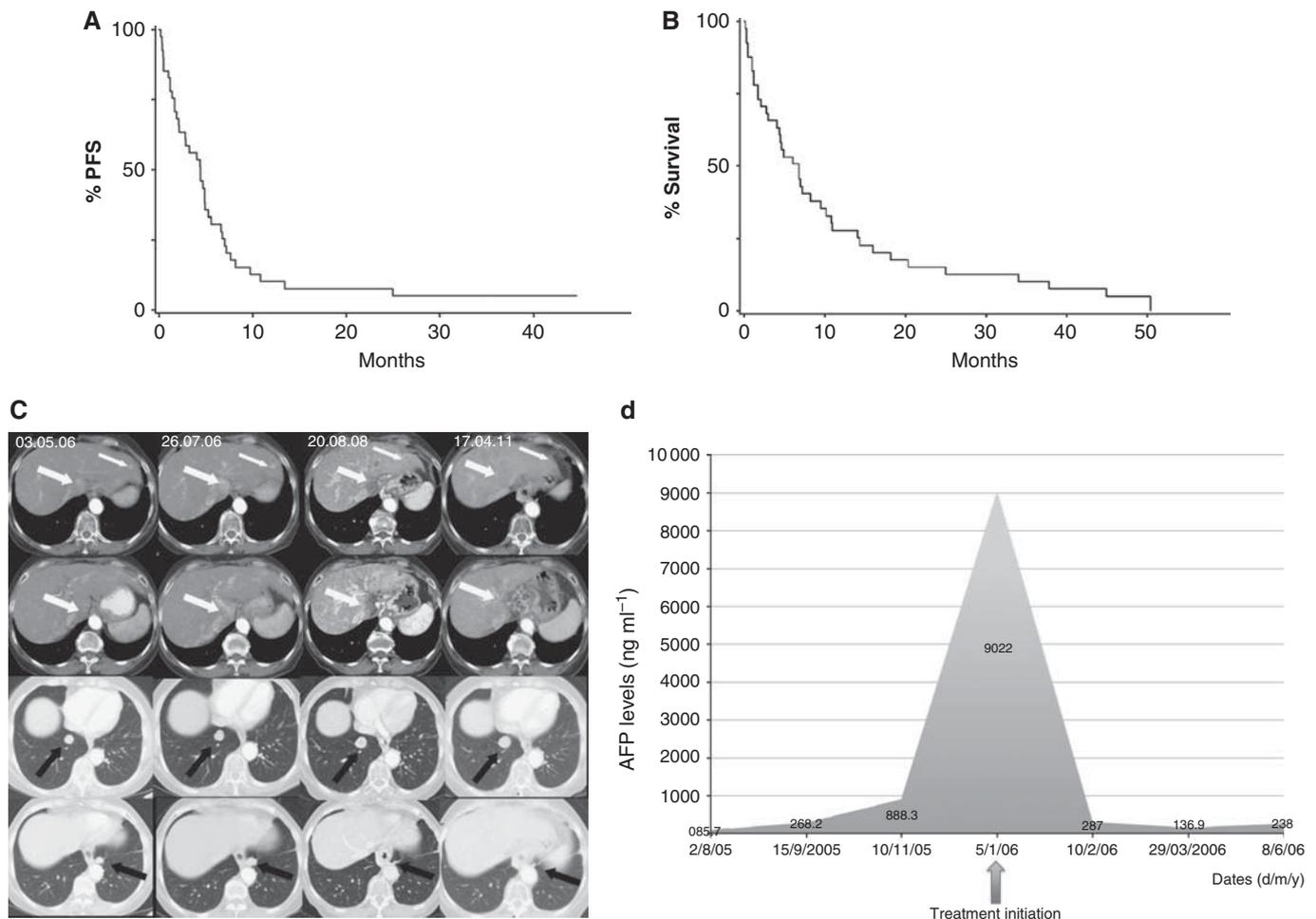


Figure 3 Progression-free and overall survival. **(A)** Median progression-free survival was 4.4 months (95% CI 2.1–5.3). **(B)** Median overall survival was 6.7 months (95% CI 3.0–10.2). **(C)** Long-term partial response in a patient with biopsy-proven hepatocellular carcinoma. A 76-year-old woman with hepatitis C and Child–Pugh A5, BCLC C, biopsy-proven hepatocellular carcinoma with bilateral pulmonary metastases, who had evidence of disease progression (+36% by Response Evaluation Criteria in Solid Tumours (RECIST) criteria) between 3 May 2006 (first column) and 26 July 2006 (second column) while enrolled in the SHARP study (Llovet et al, 2008b). Treatment with AM EMFs was initiated on 9 August 2006. Subsequent restaging multiphase contrast-enhanced computed tomographies (CTs) with images from corresponding levels (across rows) are demonstrated in the third and fourth columns over the course of 57 months. Note that the hypervascularity of the focal hepatic lesions (arrows in first two rows) became relatively hypoenhancing on arterial phase (20 August 2008). The patient developed main portal vein thrombosis with cavernous transformation as a complication of her cirrhosis. However, the intrahepatic lesion size is stable regardless of enhancement pattern. Note also that the left lung base lesion resolved (4th row), and the right lung base lesion remained stable (3rd row) over the duration of treatment. **(D)** Alpha-fetoprotein response in a 67-year-old patient with Child–Pugh A5, BCLC C HCC and hepatitis C (hepatitis B negative).

diagnosed based on elevated levels of α -fetoprotein and characteristic imaging findings such as vascular invasion and characteristic differences in tumour blood flow. One patient was excluded because liver biopsy established the diagnosis of metastatic breast cancer. Another patient was excluded because of severely impaired liver function (Child–Pugh C11). These two patients who did not meet the inclusion criteria were registered as screening failures. Hence, a total of 41 patients were eligible to receive experimental therapy (Figure 2).

Two patients were lost to follow-up as they did not come back for their scheduled appointments. Repeated efforts were made to reach the patients and their families. The date of death of only one patient is known, and no information on response to treatment is available for either patient. Four patients withdrew consent while receiving therapy after 8.0, 9.3, 20.3, and 21.0 months, respectively (Figure 2). One patient elected to receive chemotherapy, one patient had poor treatment compliance as defined by administration of less than 50% of planned treatments at two consecutive return visits, one patient

elected to enrol in another experimental protocol, and one patient requested to be considered for liver transplantation as part of an extended indication, which does not fulfil the Milan criteria (Mazzaferro et al, 1996). This latter patient experienced disease progression and was ultimately not eligible for liver transplantation. Of the 35 patients who discontinued experimental therapy, four died of gastrointestinal bleeding, three of sepsis, three of hepatic failure, one of chronic obstructive pulmonary disease, two of chemotherapy- and chemoembolisation-related complications, and one of myocardial infarction (Figure 2). The remaining 24 patients discontinued because of disease progression assessed by imaging or significant clinical deterioration as assessed by the investigator (Figure 2). Estimated 60-day mortality was 27.8%; seven of 10 deaths were directly related to progression of disease. They were caused by liver failure in association with significant hepatic tumour involvement, without other cause of death, other than tumour involvement. Two deaths were secondary to gastrointestinal bleeding. One death was due to liver failure.

A total of 31 patients (75.6%) had radiological evidence of disease progression at the time of enrolment as defined by comparison of baseline imaging studies, with imaging studies obtained within the previous 6 months; 34 (82.9%) patients had received therapy before enrolment, five (14.6%) of them systemic chemotherapy or sorafenib (Table 1). Seven (17.1%) patients had not received therapy before enrolment for the following reasons: (1) severely impaired liver function in five cases; and (2) two patients refused to receive chemotherapy for metastatic disease. As shown in Table 2, the majority of patients had severely impaired liver function as demonstrated by the fact that 22 (53.7%) patients had Child–Pugh B disease and 35 (85.4%) BLCL stage C disease.

Table 3 Independently reviewed best response (N = 41)

Best response	No.	%
Partial response ^a	4	9.8
Stable disease ^b	16	39.0
Progressive disease	8	19.5
Not available for response assessment	13	31.7

^aDuration of the partial responses were +58.0, 46.9, 14.5 and 5.3 months (patient withdrew consent to undergo liver transplant). ^bTo be classified as a stable disease, patients needed to have stable disease for ≥ 12 weeks.

Table 4 Characteristics of patients with either PR and/or long-term survival in excess of 24 months

Age at enrolment and sex	Race	Cause/cirrhosis (Child–Pugh)	Previous treatment/resection	AFP \uparrow /pathology confirmation	Extra hepatic metastasis/portal thrombosis	BCLC				Progression before study entry/response	Treatment duration/overall survival (months)	Cause of death	Treatment received after completion of experimental therapy
						Okuda	CLIP	MELD	BCLC				
62 M	Caucasian	Hep C/yes (A5)	Yes/no	Yes/yes	No/no	B	1	0	6	Yes/N/A	2.0/32.0	Tumour progressed	Systemic chemotherapy
67 F	Caucasian	Hep C/yes (B9)	Yes/no	Yes/yes	No/no	C	2	2	11	Yes/PR	11.7/11.7	GI bleed	None
30 M	Black	NOS/no	Yes/es	No/yes	No/no	B	N/A	N/A	N/A	No/PR	13.5/37.6	Tumour progressed	Chemoembolisation and systemic chemotherapy
61 M	Caucasian	Hep C/yes (A5)	Yes/no	No/no	No/no	C	1	1	6	Yes/SD	26.8/26.8	COPD	None
56 M	Caucasian	Hep B/C/yes (A5)	No/no	Yes/no	No/no	B	1	0	10	Yes/SD	4.9/50.3	Tumour progressed	Chemoembolisation
63 M	Caucasian	Hep C/yes (A5)	Yes/no	Yes/no	No/no	C	1	1	4	Yes/PR	4.9/14.3	Tumour progressed	None
76 F	Caucasian	Hep C/yes (A5)	No/no	No/no	No/yes	C	1	1	6	Yes/SD	44.6/44.6	Tumour progressed	None
76 F	Caucasian	Hep C/yes (A5)	No/yes	No/yes	Yes/yes	C	1	1	6	Yes/PR	+58.0/+58.0	On therapy	Still receiving experimental treatment

Abbreviations: AFP = α -fetoprotein; BCLC = Barcelona Clinic Liver Cancer; CLIP = Cancer Liver Italian Programme; GI = gastrointestinal; MELD = Model for end-stage liver disease; N/A = not applicable; PR = partial response; SD = stable disease.

Table 5 Changes in AFP levels

Patient age and gender	AFP 6 months (ng ml ⁻¹)	Baseline AFP (ng ml ⁻¹)	8-week AFP (ng ml ⁻¹)	AFP variation (%)	Treatment duration (months)	End treatment status	Virus status
65 M	4.31	9.76	5.95	-39.0	3.0	Progression-death	HepC
67 F	888.3	9022.0	238.0	-97.3	11.7	GI bleed-death	HepC
64 M	4.7	4.5	2.6	-42.2	8.8	AMI-death	HepB
18 M	6.7	35.7	16.4	-55.7	7.8	Revoked consent-death	NOS

Abbreviations: AFP = α -fetoprotein; AFP 6 months = AFP measured within 6 months before enrolment; AMI = acute myocardial infarction; baseline AFP = AFP at treatment initiation; GI = gastrointestinal; HepB = hepatitis B virus; HepC = hepatitis C virus; NOS = not otherwise specified; 8-week AFP = AFP at 8 weeks during treatment.

Treatment efficacy

Six of the first 23 patients (26.1%) had progression-free survival ≥ 6 months, which led us to continue enrolling patients up to the preplanned total of 41 patients (Figure 2). In total, 14 patients (34.1%) had SD for more than 6 months, which met our preplanned primary efficacy end point. Median progression-free survival was 4.4 months (95% CI 2.1–5.3) and median OS was 6.7 months (95% CI 3.0–10.2) (Figure 3A and B). One patient, previously enrolled in the SHARP study (Llovet *et al*, 2008b) and with evidence of disease progression at the time of enrolment, remains on therapy with a near complete response for 58 months (Figure 3C). Estimated survival at 12, 24 and 36 months is 27.9% (s.e. = 7.1%), 15.2% (s.e. = 5.7%), and 10.1% (s.e. = 4.8%), respectively. Subset analyses by Child–Pugh stage and accompanying figures are reported in Supplementary Information.

A total of 28 patients were evaluable for tumour response (Figure 2). Four (9.8%) patients had a partial response assessed with CT with or without contrast-enhanced ultrasound (Table 3). All partial responses were independently reviewed by two authors (MSR and DM). Three patients had biopsy-confirmed HCC and three had radiological evidence of disease progression at the time of enrolment (Table 4). Two patients had Child–Pugh A, one Child–Pugh B disease, and one had no cirrhosis. One of these

patients without biopsy-proven disease subsequently withdrew consent after 4.9 months to undergo liver transplantation. The patient died of progression of disease 9.4 months later before undergoing liver transplantation. One patient with Child–Pugh B disease had a partial response lasting 11.7 months and died of gastrointestinal bleeding. One patient died of disease progression at 44.6 months. Overall, there were six long-term survivors with an OS greater than 24 months and four long-term survivors with an OS greater than 3 years. Importantly, five of the six (83%) long-term survivors had radiological evidence of disease progression at the time of study enrolment (Table 4). Two of three patients with the longest survival (44.6 and +58 months) had radiological evidence of disease progression at the time of enrolment, BLCL stage C disease, as well as portal vein thrombosis, three predictors of short survival (Llovet *et al*, 2003). Serial AFP measurements, which predict radiological response and survival in patients with HCC (Chan *et al*, 2009; Riaz *et al*, 2009), were available for 23 patients. AFP decreased by 20% or more in four (9.8%) patients following initiation of therapy (Table 5). Figure 3D shows the time course of a 37-fold decrease in AFP in a patient who had a long-lasting (11.7 months) partial response as assessed by CT.

In all, 11 patients reported pain before treatment initiation, 3 patients reported grade 3, 5 patients reported grade 2, and 3 patients grade 1. Five patients reported complete disappearance of pain and two patients reported decreased pain shortly after treatment initiation. Two patients reported no changes and two patients reported increased pain. There were no treatment-related grade 2, 3, or 4 toxicities. The only treatment-related adverse events were grade 1 mucositis (one patient) and grade 1 somnolence (one patient) over a total of 266.8 treatment months.

DISCUSSION

Treatment with AM EMFs did not show any significant toxicity despite long-term treatment. The lack of toxicity experienced by

the 41 patients presented in this report as well as the 28 patients from our previous report (Barbault *et al*, 2009) can be readily explained by the very low and safe levels of induced RF EMFs, which are more than 100 000 times lower than those delivered during RF ablation procedures (Chang, 2003). Hence, the putative mechanism of action of this novel therapeutic approach does not depend on temperature changes within the tumour.

These data are comparable to recent phase II studies evaluating the effectiveness of standard chemotherapy as well as novel targeted therapies in HCC (Abou-Alfa *et al*, 2006; Boige *et al*, 2007; Chuah *et al*, 2007; Cohn *et al*, 2008; Dollinger *et al*, 2008; Siegel *et al*, 2008). In a large phase II study assessing the effects of sorafenib in patients with HCC and Child–Pugh A and B who had not received previous systemic treatment, Abou-Alfa *et al* (2006) observed partial responses using the WHO criteria in 2.2% of patients. Investigator-assessed median time to progression was 4.2 months, and median OS was 9.2 months. Of note, all 137 patients from that study had evidence of disease progression after 14.8 months (Abou-Alfa *et al*, 2006), whereas, at the same time point, four (9.8%) of the patients enrolled in this study did not have evidence of disease progression. These findings suggest that RF AM EMF may increase the time to radiological progression in advanced HCC.

The majority of patients enrolled in this study had either failed standard treatment options or had severely impaired liver function that limited their ability to tolerate any form of systemic or intrahepatic therapy. Indeed, 16 patients (39.0%) had Child–Pugh B8 or B9 disease. Among these patients, the median progression-free survival was 4.4 months (95% CI 1.6–7.6 months), which is identical to that of the entire group. Five of these 16 patients (31.3%) received therapy for more than 7.5 months, which indicates that this therapy is well tolerated even in patients with severely impaired liver function.

Previous treatment with standard chemotherapy or sorafenib does not seem to impact the effectiveness of AM EMFs in the treatment of HCC. Indeed, three of the four patients who had a

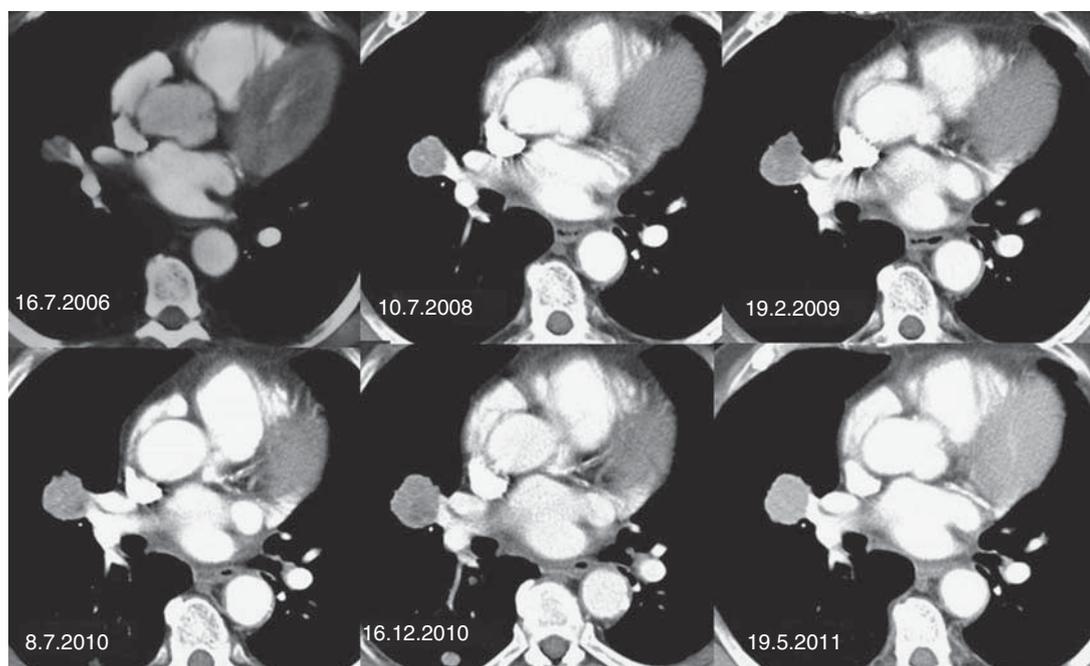


Figure 4 A 70-year-old man with recurrent thyroid cancer metastatic to the lungs: stable disease at 57.5 months. Long-term stable disease in a 70-year-old man with recurrent biopsy-proven thyroid carcinoma metastatic to the lungs enrolled in the previously published feasibility study (Barbault *et al*, 2009). Treatment with AM EMFs was initiated on 20 August 2006. As of 9 June 2011, the patient is asymptomatic and still receiving treatment with no evidence of disease progression. Images through the target metastatic lesion in the right hilum demonstrate minimal size change over the 4 years, given differences in computed tomography acquisition techniques over that time interval.

partial response while receiving AM EMFs had received previous systemic therapies (chemotherapy and sorafenib) and one had received intrahepatic therapy with ¹³¹I-lipiodol.

Tumour shrinkage as assessed by radiological imaging as well as changes in AFP levels were documented in patients with advanced HCC receiving RF EMF modulated at HCC-specific frequencies administered by an intrabuccal probe. Antitumour activity in patients with advanced HCC was exemplified by partial responses observed in four patients (9.8%) and decreases in AFP levels greater than 20% in four patients. A total of 18 patients (43.9%) either had objective response or SD \geq 6 months.

Importantly, this therapeutic approach has long-lasting therapeutic effects in several patients with metastatic cancer. Two of these patients, one with recurrent thyroid cancer metastatic to the lungs (Figure 4) enrolled in our feasibility study (Barbault *et al*, 2009) and the patient shown in Figure 3C, are still receiving treatment without any evidence of disease progression and without side effects almost 5 years after being enrolled in these studies. These findings suggest that, in some patients, this therapeutic approach may achieve permanent control of advanced cancer with virtually no toxicity.

Our phase I/II study has several limitations. First, only 19 of the 41 patients had biopsy-proven HCC, and the others were diagnosed by clinical criteria, an approach similar to that used in a recently reported phase II trial evaluating the clinical and biological effects of bevacizumab in unresectable HCC (Siegel *et al*, 2008). Importantly, analysis restricted to these 19 patients shows rates of progression-free survival at 6 months, median progression-free survival and OS that are similar to those without biopsy-proven HCC (Supplementary Figures 1C and D). Furthermore, three of the four partial responses were observed in patients with biopsy-proven HCC. Hence, these findings strongly suggest that treatment with AM EMFs yields similar results in patients with

and without biopsy-confirmed HCC. Another potential limitation of our study consists in the use of contrast-enhanced ultrasound for the monitoring of some patients with HCC. It should be pointed out that recent studies indicate that the use of this imaging technique is comparable to that of CT scan with respect to the measurement of HCC tumours (Choi, 2007; Maruyama *et al*, 2008).

Antitumour response is considered the primary end point for phase II studies to proceed to further investigations. Studies applying Cox proportional hazards analysis indicate that this end point is consistently associated with survival in trials of locoregional therapies for HCC (Llovet *et al*, 2002) and a recent consensus article suggests that randomised studies are necessary to capture the true efficacy of novel therapies in HCC (Llovet *et al*, 2008a). In summary, the encouraging findings from this study warrant a randomised study to determine the impact of AM EMFs on OS and time to symptomatic progression.

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Conflict of interest

AB and BP have filed a patent related to the use of electromagnetic fields for the diagnosis and treatment of cancer. AB and BP are founding members of TheraBionic LLC.

Supplementary Information accompanies the paper on British Journal of Cancer website (<http://www.nature.com/bjc>)

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Cancer cell proliferation is inhibited by specific modulation frequencies

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BACKGROUND: There is clinical evidence that very low and safe levels of amplitude-modulated electromagnetic fields administered via an intrabuccal spoon-shaped probe may elicit therapeutic responses in patients with cancer. However, there is no known mechanism explaining the anti-proliferative effect of very low intensity electromagnetic fields.

METHODS: To understand the mechanism of this novel approach, hepatocellular carcinoma (HCC) cells were exposed to 27.12 MHz radiofrequency electromagnetic fields using *in vitro* exposure systems designed to replicate *in vivo* conditions. Cancer cells were exposed to tumour-specific modulation frequencies, previously identified by biofeedback methods in patients with a diagnosis of cancer. Control modulation frequencies consisted of randomly chosen modulation frequencies within the same 100 Hz–21 kHz range as cancer-specific frequencies.

RESULTS: The growth of HCC and breast cancer cells was significantly decreased by HCC-specific and breast cancer-specific modulation frequencies, respectively. However, the same frequencies did not affect proliferation of nonmalignant hepatocytes or breast epithelial cells. Inhibition of HCC cell proliferation was associated with downregulation of *XCL2* and *PLP2*. Furthermore, HCC-specific modulation frequencies disrupted the mitotic spindle.

CONCLUSION: These findings uncover a novel mechanism controlling the growth of cancer cells at specific modulation frequencies without affecting normal tissues, which may have broad implications in oncology.

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Treatment of hepatocellular carcinoma (HCC) is a major challenge given the limited number of therapeutic options available (Thomas and Zhu, 2005). We have developed a novel approach to treat advanced HCC, consisting of intrabuccal administration of very low levels of radiofrequency electromagnetic fields (RF EMF), amplitude-modulated at specific frequencies, and identified using biofeedback methods in patients with cancer (Barbault *et al*, 2009). The encouraging findings from a feasibility study (Barbault *et al*, 2009) led to the design of a phase I/II trial in patients with advanced HCC, and objective responses assessed by CT-scan and changes in alpha-fetoprotein levels were observed in several patients with biopsy-proven HCC (Costa *et al*, 2011). These findings prompted us to initiate reverse translational experiments to investigate the mechanism of action of amplitude-modulated electromagnetic fields. Two different *in vitro* exposure systems operating at 27.12 MHz were used to expose cells in culture, replicating patient-treatment conditions.

Proliferation of both HepG2 and Huh7 HCC cells was significantly decreased upon exposure to radiofrequency electromagnetic

fields, which were modulated at HCC-specific modulation frequencies. To determine how such frequencies modulate cancer cell growth, we assessed differential gene expression with RNA-seq and found that the expression of several genes was significantly downregulated by HCC-specific modulation frequencies. Previous reports have shown that low intensity, intermediate frequency electric fields are capable of inhibiting cancer growth by interfering with the proper formation of the mitotic spindle (Kirson *et al*, 2004; Kirson *et al*, 2007). Similarly, we found that electromagnetic fields that are amplitude-modulated at HCC-specific frequencies disrupt the mitotic spindle of HCC cells. Thus, we provide novel evidence that very low level of amplitude-modulated electromagnetic fields block the growth of HCC cells in a tumour- and tissue-specific fashion.

MATERIALS AND METHODS

In vitro exposure devices

The design and construction of the two *in vivo* exposure devices (Figure 1) used to conduct these experiments is described in the Supplementary Information.

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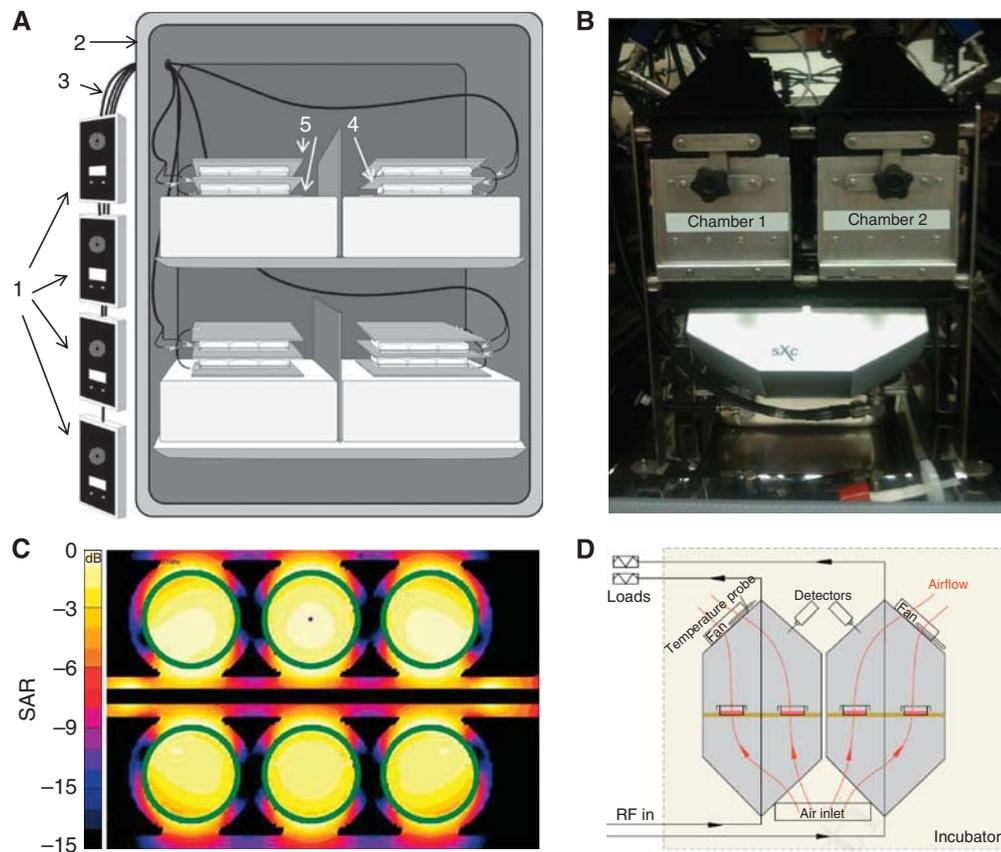


Figure 1 *In vitro* exposure experimental setups. **(A)** Parallel plate capacitor. Emitting devices (1) are placed outside the incubator (2). Each device is connected to a coaxial cable (3), which is connected to a set of brass plates inside the incubator. The centre brass plate (4) is connected to the inner conductor of the emitting device coaxial cable. The outer two brass plates (5) are connected to the outer conductor of the emitting device coaxial cable. Plates containing cells are placed in between the brass plates. **(B)** TEM cell. The system contains two identical TEM cells placed in an incubator. **(C)** Distribution of the specific absorption rate (SAR) of cell monolayer in the TEM cell (1 dB per contour). **(D)** Schematic representation showing the air flow through the TEM cell.

Cell lines

HepG2 and Huh7 cells, both of Biosafety Level 1, were used as representative HCC cell lines. HepG2 cells were obtained from ATCC (Manassas, VA, USA), and Huh7 cells were a gift from Dr Nareej Saxena (Emory University). Normal hepatocytes, THLE-2 cells, were also obtained from ATCC. The breast adenocarcinoma cell line MCF-7 was used as a representative non-HCC malignant cell line (ATCC). The breast epithelial cell line MCF-10A (ATCC) was used to represent normal breast cells. Lymphoblastoid cell lines from healthy individuals enrolled in IRB-approved protocols were provided by Dr Jeff Edberg (UAB).

[³H]thymidine incorporation assay

Growth inhibition (GI) was assessed in HCC cells exposed to HCC-specific modulation frequencies as previously described (Rosman *et al*, 2008).

Luminescent cell viability assay

Cell proliferation was quantitated using the Promega CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI, USA), a method to determine the number of viable cells in culture based on ATP quantitation.

RNA-seq

We performed RNA-seq as previously described (Reddy *et al*, 2009). We used HepG2 cells exposed to either HCC-specific modulation frequencies or to randomly chosen frequencies. We double-selected polyA-containing mRNA from 3 μ g of total RNA by using oligo-dT magnetic beads. We fragmented the mRNA with RNA fragmentation buffer and removed free-ions with a G-50 Sepharose spin column. Fragmented mRNA was used as a template to synthesise single-stranded cDNA with SuperScript II reverse transcriptase with random hexamer primers in the presence of RNaseOUT (Invitrogen by Life Technologies Corporation, Carlsbad, CA, USA). We synthesised double-stranded DNA (dsDNA) for sequencing by ligating Illumina (Illumina, San Diego, CA, USA) sequencing adaptors to blunted and dA-extended dsDNA, and size-selected fragments of 200–300 bp from a 2% Invitrogen gel and purified with a Qiagen Gel Extraction kit (Qiagen, Valencia, CA, USA). Lastly, we amplified the dsDNA library with 15 rounds of PCR with Illumina sequencing primers. Sequencing was performed on an Illumina GenomeAnalyzer IIX and the paired 36 bp reads were mapped to the hg18 reference genome by using ELAND (Illumina), allowing up to two mismatches per read and 10 or fewer map locations. By using the ERANGE software package (<http://woldlab.caltech.edu/rnaseq>), we placed uniquely mapped reads against 29 673 transcripts from NCBI build 36.1 of the human genome. After placing unique reads, ERANGE assigned multiple mapping reads and reads mapping to

splice junctions according to the number of unique reads in potential transcripts. Once all reads were mapped, ERANGE reported gene expression in units of reads per kilobase of exon and per million tags sequenced (RPKM).

Quantitative PCR

At the conclusion of the AM-EMF exposure portion of the experiment, RNA extraction (Qiagen) and reverse transcription (TaqMan, Applied Biosystems by Life Technologies Corporation) were performed to generate cDNA. Experiments comparing gene expression in HCC cells receiving HCC-specific AM-EMF with gene expression in HCC cells not receiving any exposure were conducted using Applied Biosystems pre-designed TaqMan Gene Expression Assays (*PLP2*, cat#Hs01099969_g1; *XCL2*, cat#Hs00237019_m1; Applied Biosystems by Life Technologies Corporation). Real-time quantitation was completed in quadruplicate according to the manufacturer's instructions using an ABI 7900HT Real-Time PCR System (ABI by Life Technologies Corporation), with analysis performed using ABI SDS2.2 software. Quantitative values of gene expression were determined by comparing PCR amplification curves to a known standard curve generated in tandem with the experimental samples. Each sample was individually normalised to the average corresponding to endogenous expression of *GAPDH* (*GAPDH*, cat#Hs99999905_m1, TaqMan, Applied Biosystems by Life Technologies Corporation). Averages of the normalised values from each condition were then used to compare the relative gene expression between the experimental groups. The s.e.m. was determined for each experimental condition.

Confocal laser scanning microscopy

Cells undergoing mitosis were imaged using the Zeiss LSM 710 Confocal Laser Scanning Microscope (Carl Zeiss, Inc., Thornwood, NY, USA). For imaging experiments, 22 mm square microscope cover glass (Corning Life Sciences, Lowell, MA, USA, cat#2865-22) were flame-sterilised with 200-proof ethanol and placed in 6-well or 35 mm Falcon tissue culture plates (BD Biosciences, Franklin Lakes, NJ, USA). Approximately 300 μ l of cell suspension/growth media was added directly to the top of the cover slips, and cells were plated at varying concentrations (4×10^5 – 5×10^5 cells per ml) on separate cover slips for each assay to control for variability in antibody affinity between different experiments. Once the cells were given 8–18 h to attach to the cover slips, 3 ml of complete growth media was added to each well containing a cover slip. Following RF EMF exposure, indirect immunofluorescent microscopy compared the cells receiving HCC-specific modulation frequencies with cells not receiving any exposure (Microtubule Marker (AE-8) sc-73551, Fluorescent Secondary Alexa Fluor 488 goat anti-mouse IgG (H + L): A-11001; Santa Cruz Biotechnologies, Santa Cruz, CA, USA).

Karyotype analysis

To determine whether these changes were associated with karyotypic changes, HepG2 cells exposed to HCC-specific modulation frequencies or unexposed were harvested, slides prepared, and metaphase chromosomes G-banded using standard methods. The chromosomes were analysed and the karyotype described according to the International System for Cytogenetic Nomenclature (Brothman *et al*, 2009).

Statistical analyses

One sample two-sided *t*-test was performed to test the significance of cell proliferation exposed to RF EMF amplitude-modulated at tumour-specific or randomly chosen frequencies. ANCOVA analysis: For the long-term (7 weeks) GI analysis and the GI analysis for varying SAR values (0.05, 0.1, 0.4, and 1.0 W kg⁻¹), data were fit to a linear model, and time point and dosage level were considered as covariates in determining significance.

RESULTS

Assessment of cell proliferation in the presence of RF EMF

Cell proliferation assays were conducted after 7 days, that is, 21 h of exposure to amplitude-modulated RF EMF. Treatment with HCC-specific modulation frequencies (Supplementary Table 1) significantly reduced the proliferation of HepG2 and Huh7 cells using both the parallel plate capacitor and the transverse electromagnetic (TEM) setups (Figure 1). The observed growth-inhibitory effect on HepG2 cells was of the same magnitude when using a tritium incorporation assay and a bioluminescence assay based on ATP consumption (Figure 2A). Having shown similar results with two different assays, the remainder of the cell proliferation experiments were conducted with the more commonly used tritium incorporation assay. Cell proliferation of HepG2 and Huh7 cells exposed to HCC-specific modulation frequencies was significantly lower than the proliferation of cells exposed either to randomly chosen frequencies (Supplementary Table 2) or not exposed to RF EMF (Figure 2A, columns 1–3). When HepG2 cells were exposed for only 1 h daily, we did not observe any significant inhibition of cell proliferation (Figure 2B). Daily exposure for 6 h instead of 3 h resulted in the same level of cell-proliferation inhibition (Figure 2B). To determine when HCC-specific modulation frequencies begin to exert anti-proliferative effects on HepG2 cells, we assessed cell proliferation following 3 days (9 h) of exposure and did not find any significant difference between cells exposed to HCC-specific modulation frequencies and unexposed cells (Figure 2B).

Further, to determine whether the growth-inhibitory effect of HCC-specific modulation frequencies persists over time and results in a decrease in the total number of tumour cells, we counted the number of HepG2 cells following treatment with HCC-specific modulation frequencies and that of untreated HepG2 cells weekly for up to 7 weeks. Cells that were either exposed to HCC-specific modulation frequencies or not exposed were split weekly at the same ratio over a period of 7 weeks. As shown in Figure 2C, when compared with unexposed HepG2 cells, the number of HepG2 cells following exposure to HCC-specific modulation frequencies decreased steadily over 7 weeks, resulting in a cumulative loss of 1.71×10^6 cells per ml at week 7.

The average specific absorption rate (SAR) for cells exposed in the parallel capacitor plate system is 0.03 W kg⁻¹ (Supplementary Information). All initial experiments conducted with the TEM system were conducted at a SAR of 0.4 W kg⁻¹. To determine the range of SARs within which significant GI was observed, additional cell proliferation experiments were performed at 0.05, 0.1 and 1.0 W kg⁻¹. A significant anti-proliferative effect was observed at all SARs ranging from 0.05 to 1.0 W kg⁻¹ ($P = 0.0354$). All subsequent assays with the TEM system were conducted at an SAR of 0.4 W kg⁻¹.

Inhibition of cell proliferation is tumour and tissue specific

Our previous clinical observations revealed that patients with HCC had biofeedback responses to specific modulation frequencies that were different from those identified in patients with other types of cancer, such as breast cancer (Barbault *et al*, 2009). To experimentally assess the relevance of these findings on the proliferation of tumour cells, we determined the specificity of frequencies identified in patients with these two tumour types given the documented objective clinical responses that included one complete and one partial response in two patients with metastatic breast cancer (Barbault *et al*, 2009) and three partial and one near-complete responses in four patients with HCC (Costa *et al*, 2011). A total of 194 breast cancer-specific modulation frequencies ranging in the same modulation frequency band from

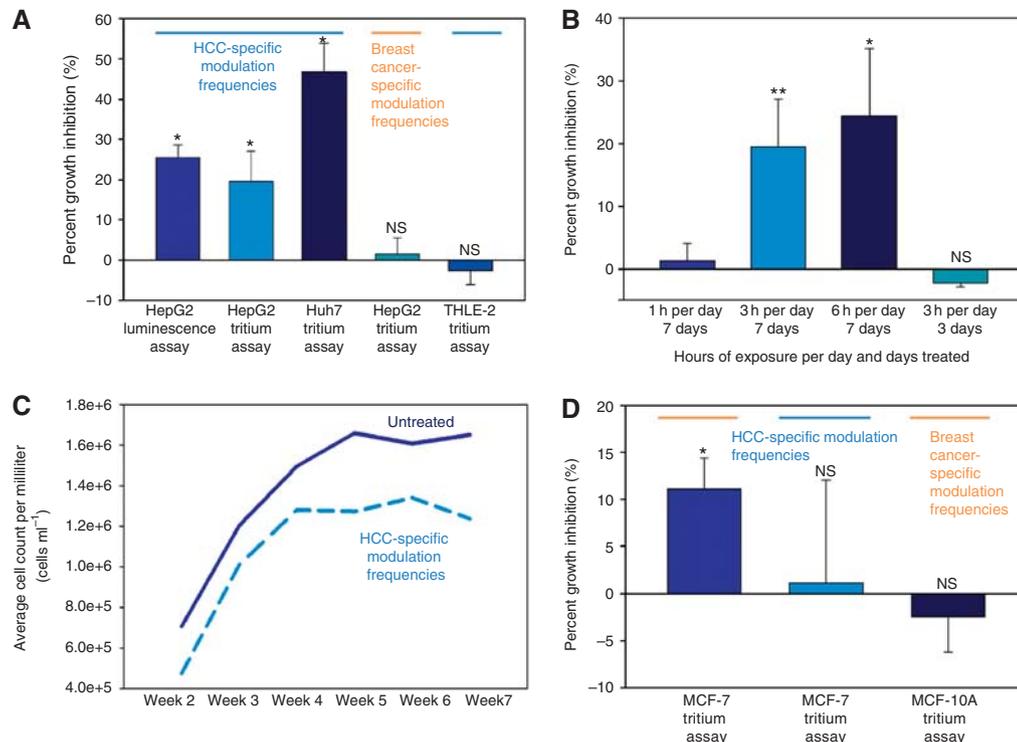


Figure 2 Cell proliferation assays of cell lines exposed to HCC-specific or breast cancer-specific modulation frequencies. **(A)** Cells were not split after initial seeding; medium was exchanged every 48 h. Experiments were performed with both equipment setups. Left to right columns: (1) HepG2 cells exposed to HCC-specific modulation frequencies with GI evaluated with a luminescence assay, $25.46 \pm 3.22\%$ GI ($P = 0.0009$). (2) HepG2 cells exposed to HCC-specific modulation frequencies with GI evaluated using tritium incorporation, $19.44 \pm 7.60\%$ GI ($P = 0.00993$). (3) Huh7 cells exposed to HCC-specific modulation frequencies, $47.73 \pm 7.14\%$ GI ($P = 0.018$). (4) HepG2 cells are not significantly inhibited when exposed to breast cancer-specific modulation frequencies, $1.49 \pm 3.99\%$ GI ($P = 0.8815$). (5) THLE-2 cells are not affected by HCC-specific modulation frequencies, $-2.54 \pm 3.54\%$ GI ($P = 0.6550$). Values represent average percent GI ($n = 6$) \pm %STERR. **(B)** Cell proliferation assays exposing cells for varying hours per day. Left to right: 1 h per day $1.36 \pm 2.77\%$ ($P = 0.8508$); 3 h per day $19.44 \pm 7.60\%$ ($P = 0.0099$); 6 h per day $24.46 \pm 10.75\%$ ($P = 0.0301$); 3 h per day for 3 days $-2.12 \pm 0.66\%$ ($P = 0.4067$). Values represent average percent GI ($n = 6$) \pm %STERR. **(C)** Cumulative decrease in cell counts over time when HepG2 cells are exposed to HCC-specific modulation frequencies. Samples were subcultured by volume every 7 days (1 : 20 split by volume). Average total cells mL⁻¹ per week: week 2: 7.07×10^5 , 4.75×10^5 ; week 3: 1.20×10^6 , 1.01×10^6 ; week 4: 1.50×10^6 , 1.28×10^6 ; week 5: 1.66×10^6 , 1.22×10^6 ; week 6: 1.61×10^6 , 1.34×10^6 ; week 7: 1.65×10^6 , 1.24×10^6 for untreated and treated samples, respectively. For the duration of the 7-week experiment with time considered as a covariate: $P = 0.005751$. **(D)** Left to right columns: (1) MCF-7 cells exposed to breast tumour-specific modulation frequencies, $11.08 \pm 3.30\%$ GI ($P = 0.0230$). (2) MCF-7 cells are not significantly inhibited when exposed to HCC-specific modulation frequencies, $1.49 \pm 3.99\%$ ($P = 0.8815$) GI, respectively. (3) MCF-10A cells are not affected by breast tumour-specific modulation frequencies, $-2.46 \pm 3.75\%$ GI ($P = 0.8579$). Values represent average percent GI ($n = 6$) \pm %STERR.

100 Hz to 21 kHz have been identified in patients with a diagnosis of breast cancer (Supplementary Table 3). In all 9 (4.6%) of the HCC-specific modulation frequencies are identical to breast cancer-specific modulation frequencies.

The two patients with metastatic breast cancer who had experienced an objective response to breast cancer-specific modulation frequencies had tumours that over-expressed oestrogen receptor (ER+) and progesterone receptor (PR+), but did not over-express ERBB2 (ERBB2-) (Barbault *et al*, 2009). We therefore chose the MCF-7 cell line as it represents the same tumour phenotype, that is, ER+, PR+, ERBB2-. Although the growth of MCF-10A breast cells was unaffected by exposure to breast cancer-specific modulation frequencies, exposure of MCF-7 breast cancer cells to breast cancer-specific modulation frequencies significantly inhibited cell proliferation (Figure 2D). However, exposure of HepG2 cells to the same breast cancer-specific modulation frequencies did not affect cell proliferation (Figure 2A). Similarly, the proliferation of MCF-7 cells was not affected by exposure to HCC-specific modulation frequencies (Figure 2D). Consequently, the observed anti-proliferative effect on HCC and breast cancer cells was observed only upon exposure to tumour-specific modulation frequencies previously identified in patients with a

diagnosis of HCC and breast cancer, respectively, despite the fact that more than 57% of the modulation frequencies only differed by <1% (Supplementary Tables 1 and 3).

Having demonstrated that the anti-proliferative effect of amplitude-modulated frequencies was tumour specific, we sought to determine whether the HCC-specific modulation frequencies have an effect on the proliferation of THLE-2 normal hepatocytes. As shown in Figure 2A, exposure of THLE-2 cells to HCC-specific modulation frequencies did not have any measurable effect on cell proliferation. These findings provide strong support for the novel notion that a combination of narrowly defined, specific modulation frequencies identified in a group of patients with the same type of cancer is capable of inhibiting cell proliferation in a tumour- and tissue-specific fashion.

Tumour-specific modulation frequencies and gene regulation

To study the mechanism by which tumour-specific modulation frequencies inhibit cell proliferation, we assessed the gene expression profile of HepG2 cells exposed to HCC-specific modulation frequencies using RNA-seq, as it provides a more

comprehensive assessment of differential gene expression across a broader range of expression levels than microarray-based analysis (Wang *et al*, 2009). Overall, we did not observe statistically significant differences in transcript levels when comparing two HepG2 cultures exposed for 1 week, 3 h a day to HCC-specific modulation frequencies with two HepG2 cultures exposed to randomly chosen modulation frequencies (Supplementary Figure 1). However, we did observe a small number of genes with an absolute fold-change >1.5 and a minimum mean RPKM of 1.5 following exposure to HCC-specific modulation frequencies. Two genes with an absolute fold-change >1.8 appeared to be down-regulated in HepG2 cells exposed to HCC-specific modulation frequencies, *PLP2* and *XCL2*, and were considered to be candidates worthy of further experiments. We validated the downregulation of *PLP2* and *XCL2* with quantitative PCR in both HepG2 as well as Huh7 cells exposed to HCC-specific modulation frequencies (Figures 3A and B). There was no significant downregulation of *PLP2* and *XCL2* in MCF-7 breast cancer cells (Figure 3C). Similarly, there was no downregulation of *PLP2* and *XCL2* in nonmalignant cells, that is, in THLE-2 normal hepatocytes (Figure 3D), or in lymphoblastoid cell lines from healthy individuals (Figures 3E and F). These findings support the novel notion that the demodulation effects of RF EMF amplitude-modulated at specific frequencies inhibit cell proliferation and affect the expression of several genes in a tumour- and tissue-specific fashion.

Tumour-specific modulation frequencies and disruption of the mitotic spindle

There is evidence that the proliferation of several rodent and human cancer cell lines is arrested by exposure to sinusoidal electric fields of $100\text{--}200\text{ V m}^{-1}$ at a frequency of $100\text{--}300\text{ kHz}$

(Kirson *et al*, 2004). This approach has also shown efficacy in animal and human tumour models as well as promising results in the treatment of patients with cancer (Kirson *et al*, 2004; Kirson *et al*, 2007; Salzberg *et al*, 2008; Kirson *et al*, 2009). The anti-tumour effect of this therapeutic approach appears to be caused by disruption of the mitotic spindle mediated by interference of spindle tubulin orientation and induction of dielectrophoresis (Kirson *et al*, 2004; Kirson *et al*, 2007). In contrast to the sinusoidal signals (Kirson *et al*, 2004), the carrier frequency of the signal applied in our experiments is more than 100 times higher; the peak E-field amplitude of the carrier at 0.4 W kg^{-1} corresponds to approximately 35 V m^{-1} inside the cell medium when the signal is sinusoidally amplitude-modulated at specific frequencies with 85% modulation depth (Kirson *et al*, 2004).

Despite these significant differences, confocal laser scanning microscopy revealed pronounced disruption of the mitotic spindle in more than 60% of HepG2 cells exposed for 1 week, 3 h per day to HCC-specific modulation frequencies whereas there was no disruption of the mitotic spindle in unexposed HepG2 cells (Figures 4A and B). Specifically, the observed cytoskeletal disruption in cells exposed to HCC-specific modulation frequencies was apparent in cells in mitosis, in which we saw centrosomal distortion and poor chromosomal separation at anaphase (Figure 4D). We found no evidence of karyotypic differences between HepG2 cells exposed to HCC-specific modulation frequencies and unexposed HepG2 cells.

DISCUSSION

By exposing HCC cells to 27.12 MHz RF EMF sinusoidally amplitude-modulated at specific frequencies, which were previously

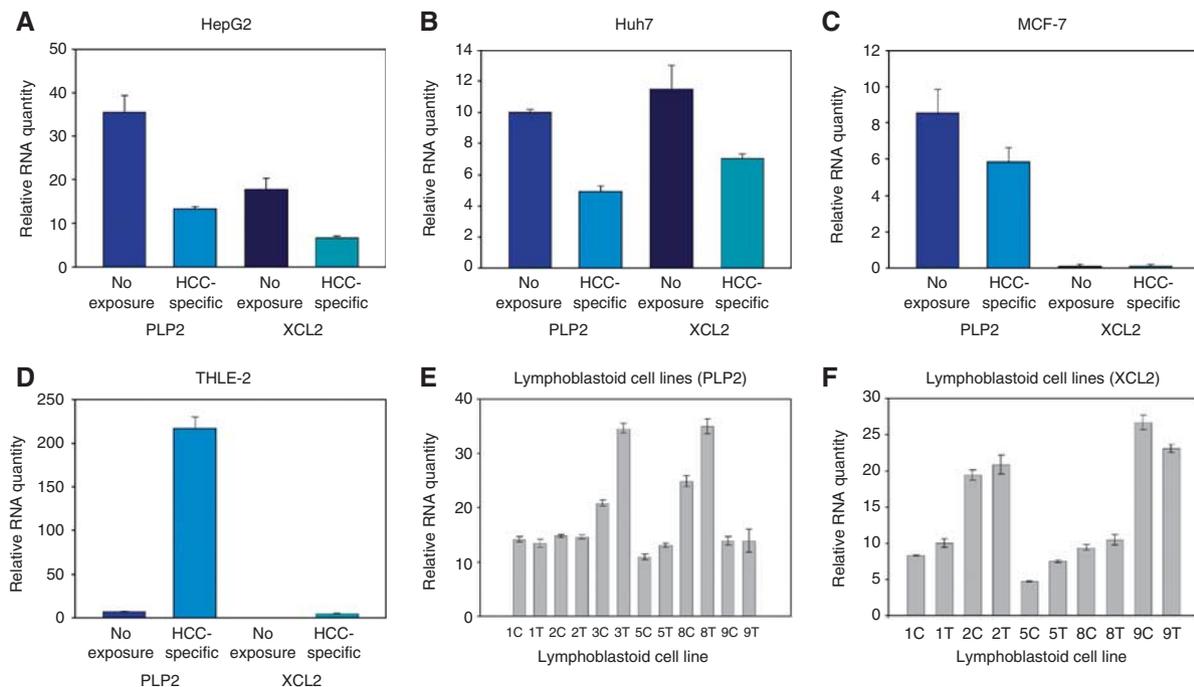


Figure 3 Expression of *XCL2* and *PLP2* receiving HCC-specific RF EMF compared with cells not receiving exposure. **(A)** HepG2: *PLP2* (35.46 ± 3.85 ; 13.17 ± 0.70) and *XCL2* (17.87 ± 2.49 ; 6.52 ± 0.48) ($P = 9.0371 \times 10^{-3}$ and $P = 0.0179$, respectively). **(B)** Huh7: *PLP2* (10.02 ± 0.19 ; 4.95 ± 0.35) and *XCL2* (11.52 ± 1.49 ; 7.02 ± 0.29) ($P = 9.4981 \times 10^{-5}$ and $P = 0.0536$, respectively). **(C)** MCF-7: *PLP2* (8.52 ± 1.30 ; 5.84 ± 0.77) and *XCL2* (levels not detectable). **(D)** THLE-2: *PLP2* (7.11 ± 0.14 ; 216.89 ± 13.18) and *XCL2* (0.03 ± 0.01 ; 4.55 ± 1.04) in THLE-2 cells exposed to HCC-specific modulation frequencies ($P = 5.5108 \times 10^{-4}$ and $P = 0.0221$, respectively). **(E)** Expression levels of *PLP2* in lymphoblastoid cell lines (C = unexposed; T = HCC-specific exposure) (for all cell lines compiled $P = 0.418$), LCL 3 expression was significant $P = 0.0021$ as was LCL 8 $P = 0.0159$ **(F)** Expression levels of *XCL2* in lymphoblastoid cell lines (for all cell lines compiled ($P = 0.899$), LCL 1 expression difference was significant $P = 0.0002$). Values represent average relative RNA expression ($n = 4$) \pm s.e.m. Levels were normalised to levels of GAPDH.

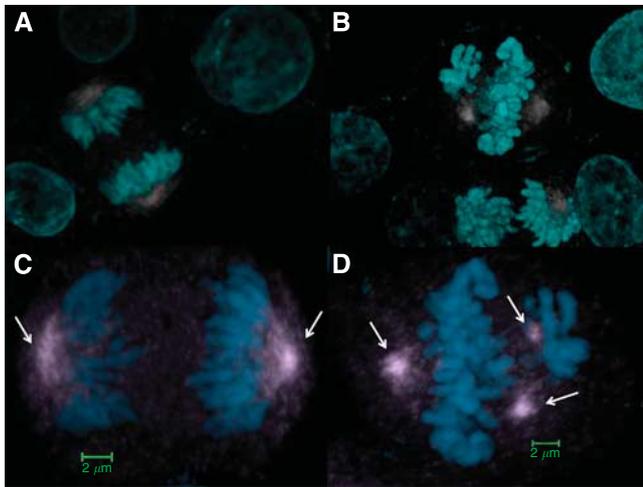


Figure 4 Mitotic spindle disruption in cells receiving HCC-specific RF EMF compared with cells not receiving exposure. **(A)** HepG2 efficiently assembles a bipolar mitotic spindle, allowing cells to pass through the mitotic assembly checkpoint and successfully progress from metaphase to anaphase. **(B)** >60% of dividing HepG2 cells exposed to HCC-specific modulation frequencies exhibit microtubule-associated anomalies. **(C)** high magnification of unexposed HepG2 cells in mitosis **(D)** high magnification of HepG2 cells exposed to HCC-specific modulation frequencies shows errors such as tripolar spindle formation (Cyan = DAPI; Gray = Microtubules; Arrows = mitotic spindle).

identified in patients with a diagnosis of HCC (Barbault *et al*, 2009) and result in therapeutic responses in patients with HCC (Costa *et al*, 2011), we demonstrate a robust and sustained anti-proliferative effect. This effect was seen within SARs ranging from 0.03 to 1.0 W kg⁻¹, that is, within the range of exposure in humans receiving treatment administered intrabuccally (Barbault *et al*, 2009; Costa *et al*, 2011). HCC-specific modulation frequencies began to hinder cell proliferation after 7 days of exposure and the anti-proliferative effect increased over a 7-week period. The anti-proliferative effect HCC-specific modulation frequencies were observed only in HCC cells, but not in breast cancer cells or normal hepatocytes.

The specificity of modulation frequencies is exemplified by the fact that two sets of similar modulation frequencies (breast cancer-specific and randomly chosen) within the same range, that is, from 100 Hz to 21 kHz, did not affect the proliferation of HCC cells. Similarly, the proliferation of breast cancer cells was affected only by breast cancer-specific modulation frequencies, but neither by HCC-specific nor by randomly chosen modulation frequencies. The fact that >50% of the modulation frequencies from these three programs differed by <1%, provides strong experimental evidence that the biological effects are only mediated by a combination of narrowly defined, tumour-specific modulation frequencies.

The modulation-frequency specific laboratory findings are consistent with the clinical observation of a complete response in a patient with breast cancer metastasis to the adrenal gland and the bone while a primary malignancy of the uterus continued to grow (Barbault *et al*, 2009). This suggests that a combination of precise tumour-specific modulation frequencies is needed to block cancer growth *in vitro* and in patients with a diagnosis of cancer. The clinical results reported by Barbault *et al*, (2009) and Costa *et al*, (2011) as well as laboratory evidence included in this report provide support for the novel and transformational concept that the growth of human tumours arising from the same primary tissue may be effectively blocked by identical modulation

frequencies. While receiving treatment with HCC-specific modulation frequencies, one black and three white patients with advanced carcinoma had partial responses (Costa *et al*, 2011). Furthermore, proliferation of the Huh7 HCC cell line, which is derived from a Japanese patient's tumour (Nakabayashi *et al*, 1982), exhibited the most pronounced response to HCC-specific modulation frequencies (Figure 2A). This indicates that the frequency signature and biological effects of HCC-specific modulation frequencies are likely independent of ethnic status.

There is no known biophysical mechanism accounting for the effect observed in these experiments; however, other modulation-frequency dependent effects have been observed in biological systems at similarly low exposure levels. Documented effects have occurred in cellular processes controlling cell growth, proliferation, and differentiation (Blackman, 2009). Further, modulation of the signal appears to be a critical factor in the response of biological systems to electromagnetic fields (Blackman, 2009). The amount of electromagnetic energy delivered is far too low to break chemical bonds or cause thermal effects, necessitating alternative mechanistic explanations for observed biological outcomes. Several theories have been put forth to explain biological responses to electromagnetic fields. Some reports have shown that low levels of electromagnetic fields can alter gene expression and subsequent protein synthesis by interaction of the electromagnetic field with specific DNA sequences within the promoter region of genes (Blank and Goodman, 2008; Blank and Goodman, 2009). Such changes have been demonstrated in the family of 'heat shock' proteins that function in the cell stress response (Blank and Goodman, 2009).

To thoroughly interrogate gene expression changes in cells exhibiting decreased cell proliferation, we used high-throughput sequencing technologies to sequence the cells' cDNA, a technique that has become invaluable in the study of cancer (Maher *et al*, 2009). Tumour cell GI was associated with downregulation of *PLP2* and *XCL2* as well as with disruption of the mitotic spindle. *PLP2* encodes an integral membrane protein that localises to the endoplasmic reticulum in epithelial cells. The encoded protein can multimerise and may function as an ion channel (Breitwieser *et al*, 1997). *PLP2* enhances chemotaxis of human osteogenic sarcoma cells (Lee *et al*, 2004) and *PLP2* downregulation is associated with decreased metastasis in a mouse model of cancer (Sonoda *et al*, 2010). *XCL2* encodes for a protein that enhances chemotactic activity for lymphocytes and downregulation of *XCL2* has been shown to be associated with good prognosis in patients with breast cancer (Teschendorff *et al*, 2007; Teschendorff and Caldas, 2008). The pronounced disruption of the mitotic spindle seen in the majority of HepG2 cells exposed to HCC-specific modulation frequencies undergoing mitosis is not associated with karyotypic changes, but may be a major determinant of the anti-tumour effects of HCC-specific modulation frequencies accounting for the therapeutic responses seen in patients receiving the same modulation frequencies (Costa *et al*, 2011).

Exposure of HCC cells to the same RF EMF modulated at slightly different modulation frequencies did not result in changes in gene expression, which demonstrates that inhibition of cell proliferation is associated with changes in gene expression levels.

In conclusion, we show that very low levels of 27.12 MHz radiofrequency electromagnetic fields, which are comparable to the levels administered to patients, inhibit tumour cell growth when modulated at specific frequencies. The exciting findings presented in this report suggest that the anti-proliferative effect of modulation frequencies is both tumour- and tissue-specific, and is mediated by changes in gene expression as well as disruption of the mitotic spindle. These findings uncover a new alley to control tumour growth and may have broad implications for the treatment of cancer.

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