

# Hepatocellular carcinoma ablation and possible immunity in the age of nanosecond pulsed electric fields

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The liver, the largest internal organ in the body, is stationed between us and everything that enters our gastrointestinal tract. Among the many roles of the liver (digestion, metabolism, storage, and production), filtration, detoxification, and immunity are major functions that shelter us from the storm of potential toxins, harmful metabolites, and microorganisms that enter the liver through the hepatic portal vein. There is sometimes a price to pay for this cleansing service, but the liver is a forgiving organ. It can regenerate itself, and when one “unit” is damaged, other units take over. The liver can suffer extensive impairment before it malfunctions and presents symptoms. These absolving liver characteristics are major causes for morbidity and mortality in liver disease. While there are hundreds of liver diseases, major ones that lead to liver cancer and, specifically, hepatocellular carcinoma (HCC) are chronic syndromes, such as hepatitis, excessive chronic alcohol consumption, metabolic syndrome, and obesity/diabetes. The single most common cause of liver disease and HCC in the USA is the hepatitis C virus (HCV), which infects nearly 4 million people.<sup>1</sup> Under such harsh circumstances, the “merciful” and tolerant liver is progressively damaged and scarred as liver cancer progresses, before warning signs appear.

Hepatocytes are the major liver cell type and are vulnerable to most forms of liver injury, either as primary or secondary insults. Continuous hepatocyte turnover by apoptosis, which is tightly coupled to inflammation and fibrosis,<sup>2,3</sup> leads to activation of stellate cells and myofibroblasts, hepatic fibrosis, and cirrhosis.<sup>4</sup> These events provide the basis for cancer-related mutations. Furthermore, such chronic proapoptotic pressure promotes the development of apoptosis evasion,<sup>5</sup> a hallmark of cancer.<sup>6,7</sup> Sustained hepatocyte turnover by apoptosis leads to fibrosis and can ultimately develop into HCC. Production of proinflammatory products from macrophages, stellate cells, and Kupffer cells during phagocytosis of apoptotic bodies appears to be responsible for the link between apoptosis and fibrosis,<sup>2,5,8</sup> a paradigm that appears to be unique to hepatocyte apoptosis.

Liver cancer is the fifth most common cancer and the third most frequent cause of mortality worldwide, with over half a million new cases every year.<sup>9</sup> HCC is the most common liver cancer, occurring in 75% of all liver cancers,<sup>10</sup> and the most frequent solid tumor worldwide.<sup>11</sup> There were approximately 33,000 new cases of liver and intrahepatic bile duct cancer in the USA in 2014.<sup>12</sup> In an analysis projecting cancer burden incidence, liver cancer was estimated to become the third leading cause of cancer-related deaths in the USA by 2030, behind lung and pancreatic

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cancer. While there were 26,000 cases of liver cancer in 2010, 47,000 and 83,000 cases are expected in 2020 and 2030, respectively.<sup>13</sup> Available studies indicate that HCV infection acquired 2–4 decades ago explain at least half of the observed increase in HCC; HCV-related HCC is likely to continue to increase for the next decade.<sup>14</sup>

HCC develops in multiple steps, often beginning with cirrhosis, progressing to adenoma and dysplastic nodule formation.<sup>15</sup> It is the liver's vital gift of continual proliferation for tissue regeneration that can ultimately be its (and our) ruin when uncorrected mutations give rise to altered stem cells (with increasing chances for additional alterations causing tumor progression).<sup>16</sup> HCC development involves mutations, epigenetic changes, noncoding RNAs, and/or translational modifications of encoded proteins that give rise to modifications in apoptosis-associated factors, oncogenes and their receptors, suppressor genes, and genes involved in cellular proliferation, cell cycle regulation, angiogenesis, and immune responses.<sup>17</sup> While HCCs are heterogeneous and many mechanistic possibilities exist, a specific scenario of tumorigenesis can include suppression of the TGF $\beta$  pathway and overexpression or activation of IL-6/STAT3 pathways.<sup>18,19</sup> Another pathway that appears to be important for inflammation-mediated tumorigenesis involves TNF $\alpha$  induction of IKK $\alpha$ -mediated phosphorylation of FOXA2, which, in turn, decreases transactivation activity toward its target gene, *NUMB*. Downregulation of *NUMB* prevents inhibition of NOTCH signaling, therefore promoting cell growth and tumorigenesis.<sup>20</sup> Epigenetic changes also play important roles in HCC development, through hypomethylation, which causes DNA instability and hypermethylation, leading to gene inactivation. For example, downregulation of E-cadherin by Snail-induced hypermethylation of the E-cadherin promoter is correlated with epithelial–mesenchymal transition, metastasis, and poor prognosis of HCC.<sup>21,22</sup> Scores of microRNAs (miRs), short noncoding RNAs that control RNA stability and translation, have been reported to be deregulated in HCC.<sup>23</sup> Downregulation of miR-199a/b-3p is commonly decreased and associated with poor prognosis. Loss of miR-122, which impairs mitochondrial function, is associated with metastasis and poor prognosis. Other factors implicated in HCC include *p53* mutations that result in genetic instability; overexpression of cyclin D1, which supports aggressive forms of HCC; and overexpression of COX-2, which affects angiogenesis, inhibition of apoptosis, and invasion and metastasis.<sup>17</sup>

It has been recommended that diagnosis and surveillance of HCC should include imaging and/or biopsy. Ultrasound has served as a common imaging diagnostic.

A definitive diagnostic for HCC has been identified as positivity of two of three stains for glypican 3, heat shock protein 70, and glutamine synthetase.<sup>24,25</sup> A search for HCC serum biomarkers that exceeds the sensitivity and specificity of AFP has identified circulating AFP isoform AFP-L3, DCP, and GP73 as useful HCC markers.<sup>26,27</sup> N-glycans, G3560, and G2890, were also identified as significant predictors of overall survival and disease-free survival, respectively, over a median follow up of 5 years. Both N-glycans also strongly correlated with other known prognostic markers, including DCP, number and size of tumors, and microscopic and macroscopic vascular invasion. The G2890 and G3560 N-glycans determined by tumor glycomics appear to be promising biomarkers for malignant behavior in HCCs.<sup>28</sup>

While development of more sensitive and specific serum biomarkers for HCC may greatly enhance early detection rates, risk assessment in treatment candidates, and identification of potential new targets for anticancer therapy,<sup>29</sup> HCC prognoses remain poor due to recurrence and/or metastasis. In recent years, nonsurgical management for unresectable HCC, such as percutaneous ethanol injection, percutaneous microwave coagulation therapy, percutaneous radiofrequency ablation, transcatheter arterial chemoembolization (TACE), chemotherapy, biotherapy, and hormonal therapy, or combinations of these have been developed. However, accepted current treatment strategies for HCC are limited, with surgery and a single approved drug, sorafenib, as options.<sup>24</sup> Clearly, new approaches are needed for HCC treatment.

Over the last decade, a novel treatment strategy has included recruitment of pulsed power technology from high-power physics for cancer treatment<sup>30–32</sup> and other applications.<sup>33,34</sup> This approach uses nanosecond pulsed electric fields (nsPEFs), which differs from conventional electroporation by using submicrosecond pulse durations instead of micro- or millisecond pulses and by using electric field strengths of kV/cm instead of V/cm. This high-power, non-thermal approach also differs from conventional electroporation by affecting intracellular structures and functions in addition to effects on plasma membranes, which is the major cellular domain of electroporation.<sup>34</sup> It also creates small, propidium iodide-impermeable pores in plasma membranes and intracellular organelles, called nanopores,<sup>35,36</sup> an effect known as supraelectroporation.<sup>37,38</sup> Preclinical trials with studies in mice and rats have shown promise with effective ablation of several tumor types,<sup>39–43</sup> including HCC.<sup>44,45</sup> Recently, the first clinical trial demonstrated efficacy for treating squamous cell carcinoma using nsPEF ablation or nanoelectroablation.<sup>46</sup> A recent finding has greatly enhanced the potential value

of nsPEFs as a cancer therapy. In addition to the efficacy of tumor ablation, rats with successfully ablated N1-S1 tumors were resistant to challenge injections of the same tumor cells, demonstrating a protective, vaccine effect<sup>45</sup> and possibly an immune response.<sup>41,45</sup> I will address this issue more specifically below.

Efficacy of cancer treatments depend on several factors. While treatment and prognosis of cancer patients are generally determined by tumor size, considerations for HCC management must also include the presence and severity of underlying diseases, such as cirrhosis or other functional maladies.<sup>47</sup> These underlying diseases, in part, account for poor responses to chemotherapeutic agents and ionizing radiation, and also account for the <20% patient eligibility for resection. The position of tumors near vital structures must also be considered in the treatment strategy.

Staging of HCC is important for diagnosis and treatment. There are several systems for tumor staging. The American Joint Committee on Cancer uses the TNM (tumor-node-metastasis) system, where disease severity is determined by tumor number and size, whether cells have invaded major blood vessels, and whether the tumor has spread to local lymph nodes or metastasized to distant lymph nodes or other organs.<sup>48</sup> Stage 0 reflects minimal involvement, usually carcinoma in-situ, whereas stage IV indicates either extensive tumor involvement or distant metastasis. The Child–Pugh Score<sup>49,50</sup> includes aspects of liver function, including blood levels of bilirubin and albumin, prothrombin time, and the presence or absence of ascites and effects on brain function.

The Barcelona–Clinic Liver Cancer (BCLC) staging classification and treatment schedule includes five tumor staging categories that consider tumor size and number, degree of liver function, and presence or absence of metastasis.<sup>51,52</sup> Classification and treatment include very early (O), early (A), intermediate (B), advanced (C), and terminal (D) stages. Stages O, A, and B are treated with resection, liver transplantation, and local treatments, such as percutaneous ethanol (or acetic acid), radiofrequency ablation, or TACE. Advanced and terminal stages are generally treated with palliative measures. As indicated above, HCCs are notoriously resistant to chemotherapeutic agents and radiation. Even the new oral multikinase inhibitor sorafenib, for advanced HCC, has had only modest clinical efficacy, extending survival by only 7–10 months.<sup>53,54</sup> At present, there are no treatment-management strategies that can effectively eliminate tumors near vital structures, avoid multiple treatments, and prevent recurrences.

These problems are mostly resolvable using nsPEF ablation; however, it must be further developed for treatment

of internal cancers by laparoscopy. Based on preclinical studies, nsPEF ablation requires a single treatment and provides sharply defined treatment zones, defined by the electrode design that surrounds the tumor mass.<sup>55</sup> In addition, the postablation vaccine effect reveals broader staging considerations for disease management. Presently considered a local treatment, it is reasonable to consider that nsPEF could be used for stages O, A, and B. Although the full extent of the vaccine effect after nsPEF ablation has not been determined, it is possible that nsPEFs could be used in advanced stages of HCC, for possible induction of an immune response.

In two HCC nsPEF-ablation studies, complete tumor ablations in an ectopic mouse model<sup>44</sup> and an orthotopic rat model<sup>45</sup> were 75% and 80%–90%, respectively. The electrode design was a five-needle array, with four grounded needles in corners of a square surrounding a high-voltage center needle.<sup>44</sup> Thus, the electric field was heterogeneous within the array, such that tumor cells were exposed to amplitudes as high as 235 kV/cm near the center needle and as low as 35 kV/cm between the needles. This can account for the observed presence of more than one cell death (CD) mechanism, since the type of CD induction is dependent on the electric field intensity (discussed later in this paper). In both studies for effective ablation, nsPEF conditions included 1,000 pulses, with durations of 100 ns and electric field strength of 50 kV/cm (determined by the voltage applied to the tumor, divided by the distance between electrodes). There were no muscle contractions. Pulse repetition rates were one per second to ensure that ablation was independent of increases in temperature. Tumors treated with less than 500 pulses frequently regrew after an initial significant reduction in tumor size and delayed slower growth.

In both mouse and rat models, the presence of cells that were positive and negative for activated executioner caspases were observed within the first 6 hours after treatment, indicating the presence of apoptotic and nonapoptotic regulated CD (RCD) mechanisms. In the rat HCC model, active caspase-9 and -3 but not caspase-8 were present, suggesting the presence of intrinsic apoptotic RCD.<sup>45</sup> However, like the mouse HCC results, not all cells expressed active caspases.<sup>44</sup> These data are consistent with *in vitro* data in Jurkat cells indicating both caspase-dependent and caspase-independent CD.<sup>56,57</sup> Others have also demonstrated multiple mechanisms of RCD in response to nsPEFs, *in vitro*.<sup>58,59</sup> A Jurkat cell *in vitro* model for nsPEF-induced CD hypothesized that Ca<sup>2+</sup> is mobilized from the external media through permeabilized plasma membranes and that Ca<sup>2+</sup>-dependent dissipation of the mitochondrial membrane potential leads to cell demise.<sup>58</sup>

However, nsPEF-induced CD mechanisms have been shown to depend on pulse conditions<sup>56,60</sup> and on cell type.<sup>59,60</sup> Consequently, not all nsPEF-induced CD mechanisms will necessarily follow this paradigm.

Perhaps more significant than the nsPEF ablation efficacy of HCC in the mouse and rat was the presence of a protective vaccine effect after successful tumor ablation.<sup>45</sup> Of 21 rats with tumors ablated for 7 weeks, none grew tumors when challenged with viable N1-S1 tumor cells, for as long as over 3 months after challenge injection and 5 months after tumor initiation. In contrast, all 24 age-matched, naïve, shipping-mate control rats required euthanasia due to tumor burden by 1 month after tumor initiation. The presence of an innate and/or adaptive immune response was supported by the massive infiltration of lymphocytes and the presence of time- and number-dependent increase in granzyme B-secreting cells 1–9 days after treatment. In a melanoma allograft system, nsPEF treatment was superior to tumor excision at accelerating secondary tumor rejection in immune-competent mice, suggesting enhanced stimulation of a protective immune response by nsPEF-treated melanomas.<sup>41</sup>

During developmental and homeostatic CD, apoptosis is anti-inflammatory and immunologically silent or tolerogenic.<sup>61</sup> However, a number of recent studies indicate that caspase-dependent processes are important for immunogenicity.<sup>61–65</sup> In chemotherapy-induced CD, some (anthracyclines) but not all (mitomycin C) caspase-inducing chemotherapeutic agents have been shown to initiate immunogenic CD (ICD);<sup>63,64</sup> there are immunogenic and nonimmunogenic subcategories of apoptosis that have yet to be differentiated. Apoptosis has been shown to induce maturation of dendritic cells, leading to T-cell activation and immunity.<sup>65</sup> Further, apoptotic cells were shown, not only to undergo degradation but also, to deliver processed antigen to dendritic cells for cross-presentation.<sup>64</sup> However, while dendritic cells are able to distinguish two types of tumor apoptotic CD, necrosis may also be able to provide factors that are critical for the initiation of immunity.<sup>66,67</sup> Autophagy and the release of adenosine triphosphate (ATP) also seem to be required for ICD.<sup>68,69</sup> ICD has obvious advantages for cancer treatment, but little is known about how death pathways influence these immune mechanisms. In the last several years, it has been realized that there is a relatively specific set of CD mechanisms that play roles in ICD. These are generally referred to as damage-associated molecular patterns (DAMPs). They include changes in cell surface membranes (externalized calreticulin binds to CD91 on dendritic cells, enhancing engulfment),<sup>70–72</sup> release of soluble factors that interact with a series of dendritic cell

receptors to enhance antigen presentation to T-cells (HMGB1 binds toll-like receptors and ATP binds to purinergic P2RX7, stimulating IL-1 $\beta$  among other DAMPs), and activation of the immune system against cancer.<sup>62</sup> Thus, increasing evidence indicates that mechanisms of tumor CD can enhance immune responses through ICD. However, whether immunogenicity depends on RCD by apoptosis, necrosis, autophagy, or all of these,<sup>73</sup> remains to be determined.

While these ICD markers have not been specifically investigated in cells exposed to nsPEFs, there are several characteristics of nsPEF-stimulated cells that suggest they are present. First, as indicated above, there are a number of studies that indicate that apoptosis induction is an important prerequisite for ICD.<sup>62,64,65</sup> In both mouse and rat HCC models, significant numbers of cells were positive for active caspases.<sup>44,45</sup> Using the five-needle electrode array in the mouse and rat HCC studies, the electric fields were heterogeneous, so cells were exposed to a relatively wide range of electric field strengths. In the mouse HCC studies, exposure of Hepa1-6 tumors to electric field intensities below 50 kV/cm resulted in delayed tumor growth, so cells near the ground electrodes in the periphery of the tumor are very likely stressed.<sup>44</sup> Some of these recover, and others likely initiate autophagy before undergoing RCD. Both a history of stress<sup>61</sup> and autophagy<sup>68,69</sup> are indicated mechanisms that promote ICD. At the other extreme, cells near the center electrode are exposed to highest electric fields, where they likely undergo necrosis, which has also been implicated as a characteristic of cells that initiate ICD.<sup>66,67</sup> The presence and absence of cells with active caspases after nsPEF treatment are consistent with these propositions. Finally, the presence of the protective, vaccine effect and the proposed immune mechanisms that are responsible for it strongly suggest that nsPEF-exposed cells present ICD markers to dendritic cells following treatments. Studies to specifically identify ICD markers and to characterize the immune cells and factors that they secrete are in progress.

Given that nsPEFs are delivered to tumors with electrodes that surround the tumor, there are some considerations as to the applicability for treatment of tumors, depending on whether they are external or internal. In all the pre-clinical studies,<sup>39–44</sup> except one,<sup>45</sup> and in the only clinical trial,<sup>46</sup> the nsPEF-treated tumors were readily accessible to needle or plate electrodes. In the orthotopic rat HCC study, tumors were treated by needle electrodes in livers exposed by laparotomy, while animals were under general anesthesia.<sup>45</sup> Future nsPEF ablation of HCC will utilize new electrode designs, currently under development, such

that treatment can be carried out under minimally invasive conditions by laparoscopy. This strategy for treating HCC and other internal organs will increase the practicality and the general availability of nsPEF ablation. It is generally considered that tumors too close to main arteries, veins, and bile ducts, and tumors that have spread throughout the liver are not resectable or treatable by other ablation methods, such as radiofrequency ablation. However, since electric fields outside the electrodes are negligible, as long as electrodes do not impale a vessel or duct, such tumors should be treatable with nsPEFs. In addition, tumors that spread throughout the liver should be treatable given the possibility for the nsPEF-induced vaccine effect and possible immune responses.

I have touched on several advantages of nsPEF ablation for treating HCC, but these benefits are worth noting in focused detail.

- First, new electrodes need to be developed that can handle high voltages, so a minimally invasive laparoscopic approach for nsPEF ablation will make it a highly effective new treatment for HCC and other internal cancers. As well, nsPEF ablation is presently ready for clinical applications treating skin cancer.<sup>39–41,43,46</sup>
- It is encouraging that nsPEFs can target multiple RCD mechanisms, including overriding apoptosis evasion, and likely evasion of immune surveillance.
- In skin cancers, it is also effective against angiogenesis and has antivasculature effects, and consequently should prevent invasion and metastasis.<sup>44,45</sup> This vascular effect appears to be different in liver, where blood flow to treated HCC tumors was shown to be transiently decreased, but recovered within at least 1 week.<sup>45</sup> The recovery of blood flow to the tumor treatment site is helpful for influxes of immune cells.
- nsPEF ablation also targets mitochondria, the seat of energy production and CD, which can lead to apoptotic and/or nonapoptotic RCD, both of which are important for ICD.
- The nsPEF treatment zone is also well-defined by the electrode design, so cancer tissues can be selectively targeted.<sup>55</sup> Because nsPEF ablation does not specifically target highly proliferative cells, it exhibits broad specificity for RCD induction in tumor masses and the tumor microenvironment, which is a site for acquiring cancer hallmarks, for tumor progression, and for establishing resistances to therapy.<sup>74</sup>
- nsPEFs can eliminate rapidly growing cells and quiescent cells, such as cancer stem cells, which could be an important factor in the heterogeneity of HCC.<sup>75,76</sup>
- nsPEF ablation has no known side effects.
- nsPEF ablation requires only a single treatment, so there is not the long exposure of tumors to chemotherapeutic agents, which that can lead to resistance-causing mutations that are often harbingers of recurrences and new cancers.
- Finally, nsPEF ablation is accompanied by a protective, vaccine effect, likely due to enhanced immune surveillance from cells undergoing nsPEF-induced CD, thereby addressing an important cancer hallmark.<sup>7</sup>

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## Disclosure

The author reports no conflicts of interest in this work.

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