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Clinical Study

Summary of bi-shRNA^{furin}/GM-CSF Augmented Autologous Tumor Cell Immunotherapy (FANG™) in Advanced Cancer of the Liver

John Nemunaitis[a–c, e] Minal Barve[a, e] Douglas Orr[d] Joseph Kuhn[f] Mitchell Magee[g] Jeffrey Lamont[d] Cynthia Bedell[a] Gladice Wallraven[b] Beena O. Pappen[b] Alyssa Roth[a] Staci Horvath[a] Derek Nemunaitis[a] Padmasini Kumar[b] Phillip B. Maples[b] Neil Senzer[a–c, e]

[a]Mary Crowley Cancer Research Centers, [b]Gradalis, Inc., [c]Medical City Dallas Hospital, [d]Baylor Medical Center, [e]Texas Oncology, PA, [f]WLS Surgical Associates, PA, and [g]Cardiovascular Specialty Associates of North Texas, PA, Dallas, Tex., USA

Key Words

FANG™ · RNA interference · bi-shRNA^{furin}/GM-CSF · Hepatocellular carcinoma · Immunotherapy · Phase I study

Abstract

Therapies for advanced hepatocellular carcinoma (HCC) are limited. We carried out a phase I trial of a novel autologous whole-cell tumor cell immunotherapy (FANG™), which incorporates a dual granulocyte macrophage colony-stimulating factor (GM-CSF) expressive/bifunctional small hairpin RNA interference (bi-shRNAi) vector. The bi-shRNAi DNA targets furin, which is a proconvertase of transforming growth factors beta (TGFβ) 1 and 2. Safety, mechanism, immunoeffectiveness, and suggested benefit were previously shown [Senzer et al.: Mol Ther 2012;20:679–689; Senzer et al.: J Vaccines Vaccin 2013;4:209]. We now provide further follow-up of a subset of 8 HCC patients. FANG manufacturing was successful in 7 of 8 attempts (one failure due to insufficient cell yield). Median GM-CSF expression was 144 pg/10^6 cells, $TGF\beta_1$ knockdown was 100%, and $TGF\beta_2$ knockdown was 93% of the vector-transported cells. Five patients were vaccinated (1 or 2.5 × 10^7 cells/intradermal injection, 6–11 vaccinations). No FANG toxicity was observed. Three of these patients demonstrated evidence of an immune response to the autologous tumor cell sample. Long-term follow-up demonstrated survival of 319, 729, 784, 931+, and 1,043+ days of the FANG-treated patients. In conclusion, evidence supports further assessment of the FANG immunotherapy in HCC.

John Nemunaitis, MD
1700 Pacific
Suite 1100
Dallas, TX 75201 (USA)
E-Mail jnemunaitis@marycrowley.org

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Introduction

Hepatocellular carcinoma (HCC) is the most common diagnosed primary liver cancer and is frequently associated with chronic viral hepatitis (hepatitis B and C) and/or cirrhosis (independent of etiology) [1]. Patients with advanced multisite disease have limited treatment options. FDA approval of the receptor tyrosine kinase inhibitor sorafenib was based on a 2.8-month improvement in survival (range 7.9–10.7 months) [2, 3]. Despite ongoing evaluation of treatment options following progression on sorafenib [4], current therapeutic efforts are limited to palliative management involving surgical resection, percutaneous ethanol injection, transcatheter arterial chemoembolization, radiofrequency ablation, or selective internal radiation [3, 5–14]. Systemic chemotherapy (i.e. doxorubicin, cisplatin, 5-fluorouracil) and/or immune therapy (i.e. interferon) has been associated with a transient response without survival advantage [15].

Although preliminary evidence supports the potential of an immunotherapeutic approach to HCC, clinical confirmation of effectiveness has been elusive. Activation of an HCC-specific response can be demonstrated through targeting of tumor-associated self-antigens, DNA-mutated antigens, or against viral antigens (hepatitis B, C virus) [16]. Clinical testing suggestive of positive activity has involved the use of adaptive transfer of lymphocytes, autologous tumor-pulsed dendritic cells (DCs) and alpha-fetoprotein (AFP) -pulsed DCs, but no significant tumor regression or survival response has been observed [17]. The use of interferon remains controversial insofar as clinical trials demonstrated reduced recurrence rates but without overall survival effect even when used in the adjuvant setting [18].

Initial phase II testing [19] results of JX-594, a nonpathogenic poxvirus modified to express a wild-type granulocyte macrophage colony-stimulating factor (GM-CSF) DNA segment, initially suggested potential benefit in advanced HCC in sequence with sorafenib. However, randomized phase IIB testing in HCC patients who progressed on sorafenib failed to demonstrate survival advantage over best supportive care [20].

The liver, however, is inherently tolerogenic [21], related, in part, to the shielding of hepatocytes by nonparenchymal structures, the abundance of local antigen-presenting cell (APC) populations [specifically Kupffer cells (KCs), liver sinusoidal endothelial cells (LSECs), and migrating DCs] [22]. One of the factors associated with immune suppression in the liver involved disease is the constitutive expression of transforming growth factor beta (TGFβ) [23, 24], which influences T cell differentiation and APC maturation. This presence, when combined with the HCC expression of TGFβ, other immune-suppressive cytokines, and molecular immune checkpoints [25, 26], further limits immune-directed, therapeutic opportunities and may relate to limited benefit, thus far demonstrated with immune-therapeutic attempts to modify HCC progression.

Recently, we have published evidence of immune induction, safety, and clinical benefit in advanced solid tumor cancer patients who received FANG™ immunotherapy [27]. FANG is a unique, triplex [17], autologous tumor cell immunotherapy, which provides three immune modulatory components: (1) an autologous whole-cell complex providing a tumor-specific full antigen matrix, (2) immune activation via local-regional GM-CSF protein expression, and (3) inhibition of $TGF\beta_1$ and $TGF\beta_2$ expression through knockdown of the proprotein convertase furin, utilizing a novel bifunctional small hairpin RNA interference (bi-shRNAi) technology [28]. In this paper, updated preliminary results of the FANG immunotherapy in the specific subset of patients with advanced HCC previously incorporated in our phase I publication [29] are reported.

Materials and Methods

The construction and cGMP manufacturing of the FANG immunotherapy have been described [27, 30]. Briefly, the FANG vector utilizes the pUMVC3 vector backbone in which the GM-CSF encoding cDNA and the DNA encoding the bi-shRNA^{furin} are under transcriptional control of the cytomegalovirus immediate early promoter of the expression vector. The final construct was confirmed by bidirectional sequencing. Following protocol-specific informed consent, the tumor was excised, placed in sterile media, and brought to the Gradalis, Inc. manufacturing facility (Carrollton, Tex., USA).

The FANG immunotherapy is manufactured over 2 conservative days by first dissociating the tumor cells into a single-cell suspension, then electroporating the FANG plasmid into the cells followed by overnight incubation. The next day, the cells were irradiated, then placed for the final fill, cryopreserved, and subjected to release testing. Following release by Quality Assurance, patients may be treated.

Study Design

The primary objective of this phase I, nonrandomized, open label trial, as previously described [27], was to evaluate the safety of the FANG immunotherapy in patients with advanced solid tumors without alternative standard therapy options. Following progression on previous therapy, the patients were entered into 1 of 3 cohorts, depending on the manufacturing cell yield from the harvested tumor, using a minimum criteria of 4 monthly injections at either 1×10^7 cells/injection (Cohort 1) or 2.5×10^7 cells/injection (Cohort 2). A maximum of 12 intradermal injections, each with a 1-ml injection volume, were administered monthly, alternating between the right and left upper arms (4 of the first 6 patients for whom doses of 2.5×10^7 cells/injection were prepared were treated with a volume of 0.4 ml to deliver 1.0×10^7 cells/injection as per FDA guidance). A safety assessment was made after the first 6 patients were administered 1.0×10^7 cells/injection. Details including image, laboratory assessment, and tumor response criteria have been previously described [27].

Eligibility requirements included the manufacturing of a minimum of 4 immunotherapy doses. The treatment was continued until documentation of progressive disease or to a maximum of 12 injections.

The trial was performed after approval by a local Human Investigations Committee and in accordance with an assurance filed and approved by the Department of Health and Human Services. This included approval for a long-term follow-up of the subset of HCC patients analyzed separately in this review.

Patient Population

All eligible patients were treated in the outpatient facilities of the Mary Crowley Cancer Research Centers (MCCRC; Dallas, Tex., USA). Specific inclusion criteria have been previously described [27].

ELISPOT Assay

The ELISPOT (enzyme-linked immunospot) assay was performed using the enzyme-linked immunospot assay for interferon gamma (BD Biosciences, San Jose, Calif., USA), as previously described [27, 31]. The reading of the ELISPOT plates was performed by ZellNet Consulting, Inc. (Fort Lee, N.J., USA). A value of ≥10 spots and >2× baseline was considered positive. ELISPOT analysis was performed on patients receiving at least 4 vaccinations, and the response status at baseline and month 4 after treatment start was compared using a paired t test (n = 18).

Results

Patient/Immunotherapy Characteristics

Eight patients with advanced HCC were entered into the phase I study (BB-IND 14205, CL-PTL 101) [27]. All underwent tumor resection as part of the standard medical management for palliative control of disease, which allowed for tumor cell procurement and FANG processing/manufacture. Patient characteristics are shown in table 1.

A successful FANG immunotherapy was constructed in 7 of the 8 patients. In 1 patient (072), insufficient tissue was available to produce the required number (4) of immunotherapies. Sufficient tissue, however, was available to test and demonstrate tumor cell viability,

Table 1. Patient characteristics

Patient ID	Age/sex	Cancer	Disease sites	Hepatitis viral status	Prior treatment
FANG-036	64/male	HCC	Liver	Negative	Therasphere, TACE, chemoembolization
FANG-039	60/male	HCC	Liver mets	Hepatitis B^{ab} positive	Doxorubicin, leucovorin, carboplatin, 5FUDR, sorafenib
FANG-044	42/female	HCC	Lung mets	Unknown	Adriamycin
FANG-047	70/female	HCC	Liver	Negative	Chemoembolization
FANG-055	40/male	HCC	Liver	Negative	Nexavar, doxorubicin, chemoembolization, intra-arterial with TACE, pankinase inhibitor, sorafenib
FANG-072	56/male	HCC	L lower lobe and lingular lung	Hepatitis C RNA PCR positive	Doxorubicin (IHA), sorafenib, doxorubicin (IHA)
FANG-073	56/male	HCC	Liver	Not done	None
FANG-078	76/male	HCC	Ascites fluid	Not done	Sorafenib, therasphere

IHA = Intrahepatic artery; mets = metastasis; L = left; TACE = transarterial chemoembolization; ab = antibody.

Table 2. Immunotherapy characteristics

Patient ID	Tissue harvested	Tissue weight, g	Dose/vial	Vials constructed, n	Mean cell viability, %	Day 7 GM-CSF expression, pg/10^6 cells	% TGFβ_1 knockdown	% TGFβ_2 knockdown	Furin knockdown PCR
FANG-036	Liver	14.20	1.0×10^7	11	97	130	94	97	Positive
FANG-039	Liver mets	13.90	2.5×10^7	7	96	61	100	91	Positive
FANG-044	Lung mets	16.28	2.5×10^7	6	95	1,520	100	73	Positive
FANG-047	Liver	63.03	2.5×10^7	8	97	158	100	48	Positive
FANG-055	Liver	74.59	2.5×10^7	9	97	55	100	99	Positive
FANG-072	L. lower lobe and lingular lung	9.00	N/A	0[a]	91	525	100	96	Positive
FANG-073	Liver	30.5	1.0×10^7	4	91	91	100	94	Positive
FANG-078	Ascites fluid	1.8 liters[b]	2.5×10^7	6	93	4,294	100	100	Positive

mets = Metastasis; L = left. [a] Minimal tissue for product assessment was available. [b] Fluid volume.

transgene expression, and the ex vivo level of knockdown of TGFβ_1/β_2 in all 8 patients compared to nontransfected autologous tumors (table 2).

Clinical Response

Five patients were treated with FANG (table 3). Three (036, 044, 047) maintained stable disease for ≥6 months, and 2 (039, 055) achieved stable disease for ≥4 months. Four of these 5 patients survived >2 years from time of treatment (table 3). No significant adverse events were observed (table 4), and immune response as measured by ELISPOT reactivity (γIFN ELISPOT assay) demonstrated a significant induction of systemic immune response in 3 of 5 of the vaccinated patients (table 5).

Discussion

This subset evaluation of FANG in patients with advanced HCC demonstrates safety and preservation of immune responsiveness (per ELISPOT) to a similar degree (3 of 5 as compared to approx. 50%) as previously reported in heavily treated cancer patients. It is encouraging

Table 3. Treatment results (as of November 21, 2013)

Patient ID	Immunotherapies received, n	Days alive since procurement	Days alive since treatment	Reason for treatment discontinuation	Current survival status
FANG-036	11	729	682	Normal completion	Dead
FANG-039	5	784	738	Disease progression	Dead
FANG-044	6	1,102	1,067	Normal completion	Alive
FANG-047	8	990	948	Normal completion	Alive
FANG-055	5	319	270	Disease progression	Dead
FANG-072	0	282	N/A	N/A	Alive
FANG-073	0	224	N/A	N/A	Alive
FANG-078	0	83	N/A	N/A	Alive

Table 4. Adverse events

Patient ID	Grade 3/4 adverse events	Serious adverse events	Relationship to FANG
FANG-036	None reported	None reported	N/A
FANG-039	None reported	None reported	N/A
FANG-044	Neutropenia	None reported	Unlikely related
FANG-047	None reported	None reported	N/A
FANG-055	Elevated alamine aminotransferase	None reported	Not related
FANG-072	N/A	N/A	N/A
FANG-073	N/A	N/A	N/A
FANG-078	N/A	N/A	N/A

Table 5. ELISPOT response

Patient ID	Baseline ELISPOT values (positive spots, n)	Month 4	Month 6	Response status[a] (+/–)
FANG-036	1	37	20	+
FANG-039	1	1	2	–
FANG-044	56	286	173	+
FANG-047	1	1	1	–
FANG-055	5	81	86	+
FANG-072	ND	ND	ND	ND
FANG-073	ND	ND	ND	ND
FANG-078	ND	ND	ND	ND

ND = Not done. [a] Response defined as ≥10 ELISPOT assay γIFN reactive spots (interpreted by a 3rd party, BD Biosciences), with ≤10 spots at baseline or more than twice the number of γIFN reactive spots at baseline.

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that all 5 treated patients experienced stable disease for ≥4 months, with 4 of these 5 surviving >2 years. In the phase III study of sorafenib, the time to radiologic progression in the control arm was 2.8 months, and the median overall survival was 7.9 months.

Long-term follow-up results of FANG in prior phase I testing show a correlation of survival with ELISPOT activation [29]. There are three key mechanisms of immune-modulating activity required for effective therapeutic activity of immunotherapy to control cancer progression. These include antigen education and/or reversal of tolerance, immune afferent arm stimulation, and suppression of innate immune inhibitors [17]. Most immunotherapies incorporate one or two of these mechanisms for induction of antitumor activity. The FANG immunotherapy, a novel class of 'triad' immunotherapies, expands the immune-modulating capability by the concurrent use of all three mechanisms. The autologous tumor tissue harvested for each individual's immunotherapy allows for the presentation of the full matrix of relevant tumor antigens. The expressive GM-CSF DNA sequence provides a multifactorial stimulus to the afferent immune recognition and processing arm of the composite response arc. Finally, the bi-shRNAi^{furin}-mediated knockdown of furin and consequent silencing of $TGF\beta_1$ and $TGF\beta_2$ expression results in the suppression of primary innate immune suppressive proteins. The latter two mechanisms, when combined with more complete antigenic exposure, allow for a higher probability of antigen (re)education and/or reversal of tolerance.

There are published data to support an immunotherapeutic approach to HCC. Tumor-infiltrating lymphocytes derived from harvested HCC samples and expanded ex vivo with interleukin-2 have been shown to lyse autologous tumors [32]. Moreover, patients with HCC in whom biopsy shows infiltration by lymphocytes have a better prognosis after surgical resection [33]. Although evidence of immune-modulating activity in HCC has also been suggested in both therapeutic [34, 35] and adjuvant settings [36–38], no randomized studies have been done in HCC to determine the effectiveness of immune-modulatory treatment [21]. The targeting of relevant cancer antigens is critical for successful cancer control through immunomodulation. AFP is expressed in 50–80% of all HCC cases. Various human leukocyte antigen (HLA)-A2- or HLA-A24-restricted AFP-specific epitopes have been identified. AFP has been shown to be an effective tumor rejection antigen in murine HCC [39]. Additionally, an AFP-derived peptide immunotherapy has been demonstrated to induce antigen-specific CD8 T cell response in HCC patients [40]. Several AFP-based immunotherapy regimens have also been reported; however, no dramatic clinical benefit was observed [6, 40–43]. GPC3, MAGE, and NY-ESO-1 are also expressed in HCC tumors, but less clinical experience is available targeting these antigens in HCC. Enhancement of immune function through cytokine stimulation, particularly IFN, has also shown some activity and/or benefit in HCC [40, 44–46], preventing or delaying tumor recurrence after surgical resection or ablation [44, 47].

However, other evidence suggests limits to immune-modulating approaches in HCC. As noted, the liver is considered an immune-privileged organ [21]. Three types of APCs are contained in the liver, namely KCs, LSECs, and DCs [22]. KCs and LSECs constitutively express the anti-inflammatory cytokines IL-10 and TGFβ [23, 24]. These immunosuppressive cytokines may play a role in immune privilege by influencing T cell differentiation and suppressing APC maturation. Hepatic stellate cells also express TGFβ after chronic liver injury [48, 49]. One of the mechanisms of tumor escape from the immune response is impairment of DC function. In cancer patients, inadequate DC function has been suggested to relate to nonresponsiveness to antitumor immunity [50]. Immunosuppressive factors that inhibit DC maturation are released by tumors; for example, human cancer cells release vascular endothelial growth factor [51]. Other cytokines derived from tumors, such as IL-6 [52] and IL-10 [53], also influence the function of DCs. Additionally, DCs have a reduced function in cancers, including HCC, in that they cannot stimulate T cells [25, 26]. HLA class I expression of HCC may be downregulated [54, 55]. However, strong HLA class I expression in HCC has also been

reported [56]. Thus, the level of major histocompatibility complex class I expression in HCC is unclear. Furthermore, expression of the co-stimulatory molecules B7-1 and B7-2 is reduced in HCC [55]. Such downregulation causes impairment of tumor antigen-processing and presentation. PD-L1 expression of KCs has been shown to be increased in tumor tissues of patients with HCC and is correlated with poor survival [57]. These data suggest that effector phase T cell inhibition is associated with tumor survival. Decoy receptor 3 (DcR3), a member of the TNF receptor superfamily, might also be involved in immune escape. DcR3 inhibits FasL-induced apoptosis by binding to its ligand Fas. Additionally, DcR3 overexpression in HCC has been reported [58, 59]. High numbers of Tregs in peripheral blood of HCC patients were detected [60, 61]. CD4+CD25+FoxP3+ Tregs impair the cytotoxic function of tumor-infiltrating CD8+ T cells [62]. The levels of the immunosuppressive cytokine IL-10 are increased in HCC patients, a finding that is related to Treg induction [63].

In conclusion, preliminary clinical and immune results as well as long-term follow-up in patients with HCC provide the basis for further disease-specific exploration of the FANG immunotherapy, warranting further testing in phase II studies.

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