**Supplementary Materials**

**Safe and Effective Sarcoma Therapy through Bispecific Targeting of EGFR and uPAR**

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**Detailed Methods for Immunohistochemistry**

Immunohistochemical staining for EGFR and uPAR in human and canine tissues was optimized for Aperio quantification as described [1,2]. Vimentin staining (Zymed cat# 18-0052 clone V9) mouse monoclonal (ThermoFisher) was used as a control for viable tissue; regions of negative staining were excluded from image analysis. CD31 staining (CD31 Dako, cat#M0823, Clone JC70A, http://www.dako.com/us/download.pdf?objectid=102467002) was used to localize the regions of tumor used for analysis and quantification. Antibody was used at a 1:100 for dilution. Heat retrieval was done prior to staining in citrate buffer (pH 6) for 30 minutes with 20 minutes cool down prior to staining. The antibodies used to stain EGFR and uPAR, respectively, were anti-EGFR precursor antibody produced in rabbit (Sigma Prestige cat# HPA018530, http://www.sigmaaldrich.com/catalog/product/sigma/hpa018530?lang=en&region=US), and clone R4 mouse anti-uPAR monoclonal antibody (Dako cat # M7294, http://www.dako.com/us/download.pdf?objectid=114115003). Methods for immunohistochemistry were the same for all antibodies.

**Detailed Description of the Canine Clinical Study**

We evaluated the safety and efficacy of adjuvant eBAT using a novel Bayesian adaptive Phase I-II trial design [3]. The primary objective of the design was to identify the biologically active (BA) dose based on a pre-defined criterion that considered the trade-off between safety and efficacy. Inference about safety and efficacy was based on the posterior distribution for the probability of 6-month survival and the probability of dose limiting toxicity (DLT) at each dose. Dose-finding was guided by a novel dose-response model that evaluated toxicity and efficacy as time-to-event outcomes, allowing new cohorts to be enrolled before previous cohorts have been fully observed and dramatically reducing trial duration. Dose-levels were considered acceptable if there was high posterior probability that the probability of toxicity was below a pre-defined threshold and the probability of 6-month survival was above a pre-defined threshold. Acceptable doses were ranked using a desirability index that considers a trade-off between efficacy and toxicity [4]. Dose-finding was completed in cohorts of three and dogs were treated at the dose that maximized the desirability index under the restriction that untried dose-levels could not be skipped during escalation. The dose that maximized the desirability index at trial completion was declared the BA dose.

Owners of each dog gave written informed consent to treat prior to study entry. All dogs were treated at the University of Minnesota Veterinary Medical Center (VMC); the study was managed by the Clinical Investigation Center (CIC) of the College of Veterinary Medicine, University of Minnesota in compliance with principles of Good Clinical Practice [5].

Inclusion criteria included histopathologically-confirmed diagnosis of stage-I (no evidence of tumor rupture) or stage-II (evidence of tumor rupture) HSA confined to the spleen; no evidence of regional or distant metastatic disease based on thoracic radiography and abdominal ultrasonography that was grossly confirmed at the time of surgery; no concurrent treatment with herbal treatments or supplements; performance score of 0 or 1 according to the Eastern Cooperative Oncology Group (ECOG) performance scale [6];adequate organ function; no serious comorbidities, such as renal or hepatic failure, congestive heart failure, or clinical coagulopathy.

Dogs were required to have a splenectomy prior to study entry. Each dog received a baseline complete history, physical examination, and pre-dose laboratory assessment that included a complete blood count (CBC), serum biochemical profile, coagulation parameters (PT/PTT) and urinalysis. Thoracic radiography and abdominal ultrasonography were performed prior to enrollment to rule out gross metastatic disease. eBAT was administered in a single cycle of three intravenous treatments at days 1, 3, and 5 at escalating doses of 25 µg/kg/day (dose level 1), 50 µg/kg/day (dose level 2), or 100 µg/kg/day (dose level 3). Cohorts of three dogs were treated at each dose level and intra-patient dose escalations were not permitted. The protocol was modified starting with the fifth dog to include pre-loading with intravenous fluids at a rate of about 0.1 to 1 ml/kg/hr for 10 to 60 minutes. The drug was administered as a slow infusion over 5-20 minutes depending on volume and size of the dog.

In total, the investigators were in contact with 181 families via email or telephone to assess their dog’s eligibility to participate in SRCBST-1. Of these, 79 dogs had surgery to remove a grossly abnormal spleen between November 28, 2012 and May 6, 2015 (51 at the VMC and 28 at another hospital prior to referral) and 23 dogs were enrolled in the study. One of these 23 dogs was euthanized at study Day 18 due to metastatic dissemination to the liver with rupture and hemoabdomen. This dog did not receive doxorubicin chemotherapy, but was included in all the analyses.

Disease reassessment included physical examination, blood and urine evaluation, thoracic radiography and abdominal ultrasonography prior to doxorubicin treatments # 3 and # 5. No medications were prescribed or administered concurrently, unless needed to manage toxicity or other, unrelated medical conditions. Adverse events related to the study drug or to doxorubicin chemotherapy were treated with supportive care, as needed. Gastrointestinal toxicities were managed with famotidine, omeprazole, metronidazole, metoclopramide, ondansetron, and/or maropitant. Antibiotic therapy was allowed for prophylaxis in the event of severe neutropenia (counts <1,000/µl) or febrile neutropenia. Non-steroidal anti-inflammatory drugs or other analgesics (tramadol, gabapentin) were allowable for pain control as needed.

Baseline characteristics for all dogs and by dose are summarized in **Table 1A**. The study protocol is summarized in **Table 1B**. The most common laboratory abnormalities at the time of screening included mild regenerative anemia (11 dogs), thrombocytosis (19 dogs), mild to moderate ALP elevation (six dogs), isosthenuria (four dogs), and slight ALT and AST elevation or slight hypoalbuminemia or proteinuria (one dog each). Slides were available for review by one of the study pathologists for 15 dogs. Most of the cases had mixed morphology, with areas showing capillary, cavernous, or solid organization. Mitotic indices also were comparable for each of these cases.

A historical comparison group (Comparison group) consisted of 28 dogs with stage-I (8 dogs) or stage-II (20 dogs) HSA treated with SOC alone (surgery followed by adjuvant chemotherapy) at the VMC between 2005 and 2014. Chemotherapy treatment in this group consisted of metronomic piroxicam and cyclophosphamide in 2 dogs, doxorubicin chemotherapy in 8 dogs, and doxorubicin chemotherapy combined with metronomic cyclophosphamide in 20 dogs. Of these 20 dogs, one was switched to a CCNU/dacarbazine regimen due to progressive disease, and in another dog metronomic chlorambucil was used to replace cyclophosphamide following the development of sterile hemorrhagic cystitis. Survival times for all dogs were calculated from the date of diagnosis to the date of death.

**eBAT Pharmacokinetics and Neutralizing Antibody Assays**

Serum samples were collected before starting treatment (time 0) and 5, 15, 30, 45, and 60 minutes after the end of the infusion on days 1 and 5 or 6 to measure drug pharmacokinetics (PK). Single serum samples were collected on days 8 and 21 to assess the presence of neutralizing antibodies (NAs). A bioassay with human RD cells was used to measure eBAT PK, as reported. Cells were incubated overnight prior to addition of the clinical batch eBAT as reported [7]. Proliferation was measured after 72 hours using a standard thymidine uptake assay. The presence of eBAT in serum was extrapolated from the standard curve as reported [8]. The presence of NAs was inferred from the capacity of serum samples to block cell death caused by reference eBAT [9].

**SUPPLEMENTARY REFERENCES**

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**Supplementary Table 1. Correlation between patient covariates and death**

|  |  |  |  |
| --- | --- | --- | --- |
| **Variable** | **N** | **HR (95% CI)** | **p-value** |
| Age (N = 212) |  |  |  |
| > 61 years | 104 | 1.66 (1, 2.74) | **0.048** |
| ≤ 61 years | 108 |  |  |
| Gender (N = 212) |  |  |  |
| Male | 95 | 1.07 (0.65, 1.76) | 0.802 |
| Female | 117 |  |  |
| Race = (N = 204) |  |  |  |
| White | 183 | 0.91 (0.33, 2.5) | 0.848 |
| Non-White | 21 |  |  |
| Tumor Volume (N = 146) |  |  |  |
| > 550 mm3 | 73 | 2.77 (1.33, 5.79) | **0.007** |
| ≤ 550 mm3 | 73 |  |  |
| Metastasis (N = 134) |  |  |  |
| Yes | 41 | 2.31 (1.23, 4.35) | **0.009** |
| No | 93 |  |  |
| Tumor Site (N = 212) |  |  |  |
| Upper abdomen or retroperitoneum | 81 | 1.42 (0.86, 2.33) | 0.17 |
| Any other location | 131 |  |  |
| EGFR (N = 212) |  |  |  |
| > 410 FPKMs | 106 | 1.69 (1.02, 2.81) | **0.043** |
| ≤ 410 FPKMs | 106 |  |  |
| PLAUR (N = 212) |  |  |  |
| > 757 FPKMs | 106 | 1.63 (0.98, 2.69) | 0.058 |
| ≤ 757 FPKMs | 106 |  |  |

\*The TCGA included samples from 59 patients with dedifferentiated liposarcomas, 2 patients with desmoid tumors, 1 patient with an undifferentiated pleomorphic sarcoma with giant cells, 105 patients with leiomyosarcomas (LMS), 9 patients with malignant peripheral nerve sheath tumors (MPNST), 25 patients with myxofibrosarcomas, 50 patients with undifferentiated pleomorphic sarcomas, and 10 patients with synovial sarcomas.

**Supplementary Table 2: Patient information (TMA)**

|  |  |  |
| --- | --- | --- |
| **Covariate** | **Units/Level** | **Mean (SD) or N(%)** |
| Age | Years | 36.9 (17.1) |
| Sex | Male | 27 (61.4) |
|  | Female | 17 (38.6) |
| Subtype | Monophasic | 29 (65.9) |
|  | Biphasic | 15 (34.1) |
| Tumor size | 5 cm or less | 14 (31.8) |
|  | Greater than 5 cm | 29 (65.9) |
|  | Unknown | 1 (2.3) |
| Lymph Node Involvement | No | 43 (97.7) |
|  | Yes | 1 (2.3) |
| Metastasis at Diagnosis | No | 41 (93.2) |
|  | Yes | 3 (6.8) |
| Nodes or Metastasis | No | 40 (90.9) |
|  | Yes | 4 (9.1) |
| Pre-Surgery Treatment | None | 32 (72.7) |
|  | Chemotherapy | 7 (15.9) |
|  | Radiation | 4 (9.1) |
|  | Chemotherapy and Radiation | 1 (2.3) |
| Chemotherapy | No | 21 (47.7) |
|  | Yes | 23 (52.3) |
| Radiation | No | 13 (29.5) |
|  | Yes | 30 (68.2) |
|  | Unknown | 1 (2.3) |

**Supplementary Table 3: Cox regression analysis for time-to-progression**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Covariate** | **Univariate Model** | | **Multivariate Model with markers only** | | **Multivariate Model with markers plus other covariates** | |
| **Hazard Ratio** | ***p*-value** | **Hazard Ratio** | **p-value** | **Hazard Ratio** | **p-value** |
| EGFR expression | 0.92 (0.76, 1.11) | 0.369 | 0.86 (0.68, 1.1) | 0.241 | 0.9 (0.71, 1.14) | 0.401 |
| uPAR expression | 1.02 (0.8, 1.29) | 0.902 | 1.13 (0.85, 1.5) | 0.397 | 0.95 (0.68, 1.33) | 0.775 |
| Age |  |  |  |  | 1 (0.96, 1.03) | 0.903 |
| Sex (reference = Male) |  |  |  |  | 0.64 (0.19, 2.14) | 0.464 |
| Subtype (reference = monophasic) |  |  |  |  | 1.12 (0.36, 3.51) | 0.848 |
| Tumor Size (reference = 5 cm or less) |  |  |  |  | 3.2 (0.84, 12.25) | 0.089 |
| Nodes or Metastasis (reference = no) |  |  |  |  | 4.04 (0.41, 40.26) | 0.234 |
| Chemotherapy (reference = no) |  |  |  |  | 0.49 (0.11, 2.11) | 0.336 |
| Radiation (reference = no) |  |  |  |  | 0.7 (0.22, 2.29) | 0.558 |

**Supplementary Figure 1. Survival probability based on *EGFR* expression**

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P=0.043

Supplementary Figure 1. Survival probability based on *EGFR* expression. Graph illustrating death events in patients where EGFR expression was in the upper 50th percentile versus patients with EGFR expression in the lower 50th percentile.

**Supplementary Figure 2. Quantification of EGFR and uPAR expression in normal canine tissues and in canine hemangiosarcoma**

**A**

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**B**

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Supplementary Figure 2. Quantification of EGFR and uPAR expression in normal canine tissues and in canine hemangiosarcoma. Box and whisker plots summarizing EGFR (A) and uPAR (B) expression in a tissue microarray containing kidney, liver, and spleen tissues from normal dogs, tumor samples from 15 dogs in the SRCBST-1 study, and samples from two dogs with focal splenic hematomas.

**Supplementary Figure 3. CONSORT diagram for SRCBST study**

Total enrolled (n=23)

Euthanized prior to doxorubicin due to metastasis and hemoabdomen (n=1)

Safety and toxicity assessment (n=23)

Follow-up >180 days until death (n=14)

Excluded

Benign splenic lesions or non HSA tumors, excluded (n=31)

Splenic HSA

(n=20)

* Metastasis at surgery, excluded (n=10)

Screened (n=10)

* Declined screening, excluded (n=4)
* Screened (n=6)
  + Metastasis, excluded (n=1)

Splenic HSA

(n=28)

Screened (n=28)

Concurrent cardiac disease, excluded (n=1)

Declined study participation, excluded (n=2)

Metastasis, excluded (n=7)

Enrolled (n=5)

Enrolled (n=18)

Splenectomy at the VMC

(n=51)

Splenectomy at rDVM, referral to the VMC

(n=28)

Initial Contacts (n=181)

Excluded from initial contact (n=102)

* Did not meet inclusion criteria (n=50)
* Declined participation (n=52)

Alive <180 days (n=9)

Supplementary Figure 3. Enrollments, exclusions, and assessments. Flow chart with details of dogs enrolled in the study and exclusions from each of the measured endpoints.