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Predictive value of *ex vivo* biodynamic imaging in determining response to chemotherapy in dogs with spontaneous non-Hodgkin's lymphomas: a preliminary study

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Abstract

Biodynamic imaging (BDI) is a novel phenotypic cancer profiling technology which optically characterizes changes in subcellular motion within living tumor tissue samples in response to *ex vivo* treatment with cancer chemotherapy drugs. The purpose of this preliminary study was to assess the ability of *ex vivo* BDI to predict *in vivo* clinical response to chemotherapy in ten dogs with naturally-occurring non-Hodgkin's lymphomas. Pre-treatment tumor biopsy samples were obtained from all dogs and treated *ex vivo* with doxorubicin (10 μ M). BDI measured six dynamic biomarkers of subcellular motion from all biopsy samples at baseline and at regular intervals for 9 h following drug application. All dogs subsequently received doxorubicin to treat their lymphomas. Best overall response to and progression-free survival time following chemotherapy were recorded for all dogs. Receiver operating characteristic (ROC) curves were used to determine accuracy and identify possible cut-off values for the BDI-measured biomarkers which could accurately predict those dogs' cancers that would and would not respond to doxorubicin chemotherapy. One biomarker (designated 'MEM') showed 100% discriminative capability for predicting clinical response to doxorubicin (area under the ROC curve = 1.00, 95% CI 0.692–1.000), while other biomarkers also showed promising predictive capability. These preliminary

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findings suggest that *ex vivo* BDI can accurately predict treatment outcome following doxorubicin chemotherapy in a spontaneous animal cancer model, and is worthy of further investigation as a technology for personalized cancer medicine.

Keywords

personalized medicine; phenotypic profiling; cancer; animal model

Introduction

Personalized cancer medicine (PCM) aims to predict the response of individual patients' cancers to chemotherapy, historically relying upon detailed genotypic analyses of tumors to identify molecular targets for drug therapy [1, 2]. While this approach has yielded some remarkable successes, several challenges still limit the routine use of PCM in the clinic. For one, cancer genomes are highly complex and chaotic, thus genotypic aberrations may not completely predict phenotypic behaviors such as clinical response to drug therapy [1]. In addition, genomic approaches may not adequately model the signaling network existing among cancer cells or between cancer cells and stromal cells within the tumor microenvironment, yet these cell-cell interactions exert a powerful influence upon drug response [3]. A PCM assay which characterizes the phenotypic response of a cancer to drug therapy *a priori*, while recapitulating cell-cell communications within the tumor at a 3D tissue level, would be highly desirable for overcoming these limitations.

Biodynamic imaging (BDI) is a novel live-tissue imaging technology that addresses many of these limitations to current PCM approaches. BDI analyzes temporal fluctuations in subcellular motion by recording dynamic patterns of short-coherence infrared light scattering at a depth of up to 1 mm within living, 3D tumor tissues [4–9]. BDI has been used previously to characterize subcellular motion in cultured 3D tumor cell spheroids and *ex vivo* biopsy samples collected from murine tumor xenografts. An *et al* showed that BDI identifies disparate phenotypic drug responses in tumors derived from drug-resistant and drug-sensitive cell lines. For instance, when tumor biopsies harvested from murine tumor xenografts were treated *ex vivo* with cisplatin, BDI observed a greater reduction in aggregate subcellular motion in tumors derived from the platinum sensitive A2780 ovarian carcinoma line than in tumors derived from the platinum-resistant A2780-CP70 line [6]. In addition to measuring time-dependent changes in aggregate subcellular motion within tumor tissues, BDI also assesses finely graded aspects of subcellular motion, measured across varying frequencies, that correspond to different physiologic processes within the cell. For example, large-scale motion, such as that associated with membrane blebbing during apoptosis, is detected at lower frequencies, whereas small-scale motion, such as that associated with internal organelle movements, is detected at higher frequencies [7, 8]. These various motions are captured graphically as a drug response spectrogram, which can be used to segregate drug-sensitive from drug-insensitive tumors, while also characterizing a drug's molecular mechanism of action [7–9].

While BDI has successfully characterized drug responses in cultured tumor spheroids and murine tumor xenografts, it has not previously been applied to predicting treatment outcome in a spontaneous animal tumor model. Naturally-occurring non-Hodgkin's lymphomas (NHL) in dogs represent a highly suitable preclinical animal tumor model in which to evaluate the predictive power of BDI. Non-Hodgkin's lymphomas are common tumors in dogs, with histopathologic, molecular, and clinical features strikingly similar to NHL in humans [10]. Generalized peripheral lymphadenomegaly is the hallmark of most NHL in dogs, although liver, spleen, and bone marrow involvement are also common. Doxorubicin-based combination chemotherapy is the standard of care for dogs with NHL, but the goal of treatment is to afford durable cancer remission and long-term disease palliation, while preserving quality of life, rather than to cure the cancer. Important clinical endpoints can be assessed rapidly in dogs with NHL—best overall response (BOR) to chemotherapy typically is evident within days following treatment, and progression-free survival time (PFST) after chemotherapy is approximately 4–9 months [11]. Moreover, as is the case with human cancers, spontaneous NHL in dogs are both biologically and clinically diverse, thus both BOR and PFST following chemotherapy vary dramatically from dog to dog. This heterogeneity in response to therapy, in particular, makes NHL in dogs an excellent model in which to investigate BDI as a predictive assay for PCM. The purpose of this preliminary study was to determine the extent to which BDI, performed upon tumor biopsies obtained from dogs with NHL and treated with doxorubicin *ex vivo*, predicts BOR and PFST following doxorubicin chemotherapy in the same dogs *in vivo*.

Methods

Study animals

Ten dogs with untreated, naturally-occurring NHL were prospectively enrolled into this study. All animals were privately-owned pet dogs seen at the Purdue University Veterinary Teaching Hospital between August 2013 and March 2014. The study protocol was approved by the Purdue Animal Care and Use Committee, with written informed consent obtained from the owner of each dog prior to enrollment. Study eligibility was based upon a clinical presentation consistent with primary nodal NHL and preliminary diagnosis of intermediate-to-high grade lymphoma made by fine needle aspirate cytology of an affected lymph node. Additional criteria necessary for inclusion in the study were: body weight >15 kg, presence of at least one peripheral lymph node with longest diameter ≥ 2.5 cm, and expected survival of ≥ 4 weeks with treatment. Dogs were excluded if any of the following were present: primary extranodal NHL, neutrophils <2 500/ μL^{-1} , platelets <100 000/ μL^{-1} , clinically significant cardiac dysfunction (defined as any ventricular arrhythmia, atrioventricular block, cardiomyopathy, congestive heart failure, or other condition which would reasonably preclude doxorubicin treatment), clinically significant hepatic dysfunction (defined as serum alanine aminotransferase activity ≥ 4X upper limit of normal, hyperbilirubinemia, or serum biochemical evidence of hepatic synthetic failure), or prior treatment of any kind for the lymphoma.

Clinical staging and treatment of study animals

At the time of study enrollment, all dogs underwent surgical biopsy of an affected peripheral lymph node to provide material for histopathologic confirmation of lymphoma and BDI. Nine dogs underwent incisional wedge biopsy of an affected lymph node, while one dog underwent multiple core biopsies using a 12 gauge biopsy needle (Magnum[®] disposable needle (12 G × 10 cm), Bard Medical). A portion (approximately 125 mm³) of each dog's biopsy was transferred to RPMI 1640 cell culture medium (Mediatech, Inc.) and submitted for BDI, while the remainder was fixed in 10% neutral buffered formalin and submitted for histopathologic evaluation. All lymphomas were histopathologically subtyped according to World Health Organization (WHO) criteria [12], based upon histomorphology in hematoxylin and eosin-stained tissue sections and immunohistochemical detection of CD3 or CD79a, as previously described [13]. All dogs underwent standardized cancer staging tests, including complete blood count, serum biochemistry profile, thoracic radiography, abdominal ultrasonography, bone marrow aspirate cytology, and electrocardiogram, and were assigned a tumor stage based upon WHO criteria [14]. Following completion of staging tests, all dogs were scheduled to receive single-agent doxorubicin at a dose of 30 mg m⁻² administered intravenously once every 3 weeks for a maximum of 5 doses. While combination chemotherapy is considered standard for dogs with nodal NHL, single-agent doxorubicin treatment is a well-accepted and efficacious alternative to combination drug therapy [15, 16].

Assessment of clinical endpoints

Caliper-based measurement of peripheral lymph nodes was used to assess BOR to chemotherapy, in accordance with criteria established by Vail *et al* [17]. Briefly, complete remission (CR) was defined as the absence of measurable tumor burden; partial remission (PR) was defined as 30% reduction in the sum of the longest diameters of measurable tumor lesions; progressive disease (PD) was defined as 20% increase in the sum of the longest diameters of measurable tumor lesions, or the appearance of new lesions; and stable disease (SD) was defined as measurable tumor burden that was neither PR nor PD. Best overall response was assessed once every three weeks during the course of treatment, and then once monthly following completion of treatment. Exceptions to this protocol were allowed if rapid disease progression necessitated prompt medical attention during the interval between scheduled rechecks. Progression-free survival time was defined as the time elapsed between administration of the first doxorubicin treatment and the first detection of PD, or death due to any cause, whichever came first. All dogs were considered off study at the time that PD was first detected or at the time of death. Given that treatment outcome of canine NHL is strongly dependent upon the ability of chemotherapy to induce PR or CR early in the course of therapy, owners of dogs experiencing SD following the first dose of doxorubicin were given the option to withdraw their dog from the study in order to pursue alternative treatment options.

Biodynamic imaging

At the time of study enrollment, a portion of each dog's surgical biopsy was placed in RPMI 1640 and transported immediately to a nearby laboratory for BDI. Each dog's biopsy was

processed into approximately 16 individual tissue samples of approximately 1 mm³ size. These samples were mounted in 8-well chamber slides (Nunc Lab-Tek), with each well containing several tumor samples. A thin layer of low-gel temperature porous agarose (Sigma-Aldrich) was used to immobilize the tumor samples within the wells, and all samples were then overlaid with RPMI 1640 containing 10% fetal bovine serum (Atlanta Biologicals), 100 U mL⁻¹ penicillin, 0.1 mg mL⁻¹ streptomycin, and 25 mM HEPES [6]. BDI was subsequently performed on all tumor samples from each dog using previously described methods [4–9]. A schematic diagram of the BDI instrument is provided in supplemental figure S1 (stacks.iop.org/CSPO/1/015003/mmedia). The BDI system is a short-coherence Mach Zender interferometer with digital holographic acquisition and reconstruction. The 3D capabilities are provided by coherence-gated detection that is equivalent to laser ranging using time-of-flight detection of backscattered light. When light scatters from subcellular motion, it acquires a Doppler frequency shift proportional to the speed of the scattering objects (e.g. mitochondria, vesicles, nucleus, cell membrane). Because of the multiple scattering objects moving inside cells, the Doppler frequencies are detected as time-varying fluctuations in the detected intensities. The frequencies of these fluctuations relate directly to the speeds of the scattering objects, and the amplitudes of the fluctuations relate to the overall activity of cellular processes.

Baseline BDI data were recorded from all samples from each dog's tumor for approximately 4 h, then doxorubicin (10 μ M) was applied to all samples. The concentration of doxorubicin applied is comparable to that which is achievable in plasma following intravenous administration of clinically relevant doses of doxorubicin to dogs [18, 19]. Following *ex vivo* doxorubicin treatment, BDI was performed at regular intervals on all samples for approximately 9 h. *Motility contrast images* [5, 6, 8] and *drug response spectrograms* [7–9] were generated from all tumor samples using BDI. Motility contrast images are spatial maps of the amplitudes of the temporal fluctuations at a fixed depth inside the tumor tissue set by the coherence gate (typically at a depth of 400 microns inside the sample). Subcellular motion, as depicted in motility contrast images, is measured in terms of the *normalized standard deviation (NSD)* of each pixel in the image. The NSD of a given pixel in the motility contrast image is calculated as the standard deviation in the fluctuating intensity divided by the average intensity of that pixel [5, 6]. Drug response spectrograms are time-frequency representations of the change in fluctuation spectral content in response to an applied drug. The frequency range in the spectrograms spans from 0.01 Hz to 12.5 Hz, representing the range of motion from whole-cell shape changes (lowest frequencies) to membrane motions (mid frequencies) and vesicle transport (highest frequencies). Examples of motility contrast images and drug response spectrograms, with notes on their interpretation, are provided in the supplemental materials, figure 1, figure 3, and supplemental figure S2 (stacks.iop.org/CSPO/1/015003/mmedia).

Dynamic biomarkers of subcellular motion measured by BDI included: (1) *NSD*, which describes baseline (i.e. prior to treatment with doxorubicin) aggregate subcellular motion captured in motility contrast images, averaged over time; (2) *change in NSD from baseline (DNSD)*, which describes the change relative to baseline in aggregate subcellular motion captured in motility contrast images, averaged over time, following *ex vivo* doxorubicin treatment; (3) *blue tail (BLUETAIL)*, which describes the extent to which marked reduction

in subcellular motion was apparent at any frequency in any of the drug response spectrograms from each dog's tumor samples; (4) *mid-frequency assessment (MEM)*, which describes changes in motion apparent within the middle frequency range (0.1–1 Hz) in drug response spectrograms; (5) *non-linear frequency assessment (APOP)*, which describes changes in motion occurring at both high (>1 Hz) and low (<0.1 Hz) frequencies in drug response spectrograms, and for which specific spectral patterns have previously been correlated with an apoptotic response [8, 9]; and (6) *all frequencies (FULLSPEC)*, in which data from the full frequency range (0.01–12.5 Hz) in drug response spectrograms are considered collectively. An explanation of how changes in the values of these biomarkers are reflected in motility contrast images and drug response spectrograms is provided in supplemental figures S3–S4 (stacks.iop.org/CSPO/1/015003/mmedia).

To account for intratumoral heterogeneity in subcellular motion in each dog's tumor, data from multiple processed biopsy samples (ranging from 8 to 32 samples from each dog's tumor) were averaged to generate mean biomarker values for each dog. Mean biomarker values were used for all subsequent statistical analyses. A visual representation of the effect of averaging data from several samples of a tumor biopsy from a single dog is provided in supplemental figure S4 (stacks.iop.org/CSPO/1/015003/mmedia).

Statistical analysis

Descriptive statistics, including BOR to chemotherapy and PFST, were recorded for each dog. For the purposes of analysis, BOR was dichotomously classified as either response (CR) or non-response (PR, SD, PD) to doxorubicin. The mean values of the six biomarkers of subcellular motion (NSD, DNSD, BLUETAIL, MEM, APOP, and FULLSPEC) were analyzed using receiver operating characteristic (ROC) curves. The accuracy of each biomarker was assessed by calculating the area under the ROC curve (AUC) and its 95% exact binomial confidence interval. The ROC curves were also analyzed to identify possible cut-off values for each biomarker which would differentiate responder from non-responder dogs.

Results

Eight dogs with B-cell NHL and two dogs with T-cell NHL were enrolled. Seven of the eight dogs with B-cell NHL had diffuse large B-cell lymphoma (DLBCL), while in the eighth dog, WHO subtyping was not performed because the biopsy method (needle core) provided inadequate tissue for this purpose. Both dogs with T-cell NHL had peripheral T-cell lymphoma, not otherwise specified (PTCL-NOS). Doxorubicin chemotherapy was tolerated well by all dogs, and treatment-related adverse events generally were mild, in keeping with previous reports [15, 16]. Demographic and treatment response data are summarized in table 1. Best overall response to doxorubicin was classified as response in 6 dogs experiencing CR of their NHL and non-response in 4 dogs experiencing PR (1), SD (2) or PD (1). Ultimately, 8 of the 10 dogs died of lymphoma. One responder dog died of metastatic hemangiosarcoma 210 d following induction of doxorubicin chemotherapy. A complete post-mortem examination of this dog identified no evidence of lymphoma. One responder dog was lost to

follow-up 176 d following induction of doxorubicin chemotherapy with its NHL still in complete remission at that time.

Representative averaged motility contrast images from two study dogs are depicted in figure 1. In dogs whose lymphoma was responsive to doxorubicin *in vivo*, many of the tumor biopsy samples showed a dramatic reduction in aggregate subcellular motion following *ex vivo* application of doxorubicin. In contrast, minimal change in aggregate subcellular motion following *ex vivo* doxorubicin treatment was observed in the majority of biopsy samples from dogs whose cancers which were non-responsive to doxorubicin *in vivo*

Figure 2 depicts aggregate subcellular motion within tumor biopsies as a function of time for the responsive and non-responsive tumor populations. The mean change in NSD from baseline (i.e. 'Delta NSD' or 'DNSD') for tumors from responsive dogs was DNSD = -0.055 , and that for tumors from non-responsive dogs was DNSD = -0.013 . The DNSD values were evaluated from the end of the experiment, even if the NSD values had not stabilized to a final steady value. A faster response was reflected in a larger DNSD evaluated at a fixed time after application of doxorubicin, hence DNSD can capture both the magnitude and speed of response. Following *ex vivo* application of doxorubicin, a marked drop in NSD from baseline (i.e. large negative DNSD value) was apparent in tumors from dogs experiencing CR of their NHL to doxorubicin *in vivo*. In contrast, NSD did not deviate substantially from baseline in tumors from dogs experiencing PR, SD, or PD of their NHL following doxorubicin chemotherapy.

Representative averaged drug response spectrograms from two study dogs are depicted in figure 3. Panel A of the figure depicts an averaged drug response spectrogram in which motions at all frequencies are essentially unchanged or slightly enhanced following *ex vivo* application of doxorubicin; this spectrogram corresponds to a dog that experienced PD (non-response) of its NHL following doxorubicin treatment *in vivo*. Panel B depicts an averaged drug response spectrogram in which motions at all recorded frequencies are significantly suppressed following *ex vivo* application of doxorubicin; this spectrogram corresponds to a dog that experienced CR (response) of its NHL following doxorubicin treatment *in vivo*.

Dot plots depicting the distributions of the six BDI-measured biomarker values for the 10 study dogs are presented in figure 4. The raw data used to generate these dot plots are presented in supplemental table S1 (stacks.iop.org/CSPO/1/015003/mmedia). Analyzing these biomarker values using ROC curves showed that the MEM biomarker had the greatest ability to predict response versus non-response of these dogs' cancers to doxorubicin chemotherapy. A cut-off value of -0.054 for this biomarker correctly classified dogs as responders or non-responders in 100% of cases (area under the ROC curve = 1.00, 95% CI 0.692–1.000). The areas under the ROC curves for several other motility biomarkers, including APOP, FULLSPEC, BLUETAIL and DNSD, also were high (table 2), indicating that these biomarkers had good-to-excellent power to discriminate responders versus non-responders in this population of dogs.

Discussion

In this preliminary study, we showed that *ex vivo* BDI can predict with high accuracy the *in vivo* response to chemotherapy in a naturally-occurring animal model of non-Hodgkin's lymphoma. All prior investigations of BDI have involved the use of established tumor cell lines cultured as 3D spheroids or xenografted to murine hosts. In these prior investigations, various aspects of tumor cell motion measured by BDI correlated well with known properties of the cell lines under study, such as proliferative rate and sensitivity or resistance to chemotherapeutic drugs [5–9]. The present study, however, marks the first time that BDI has been shown to predict clinical response to chemotherapy in a mammalian species undergoing treatment for a spontaneous cancer. To the authors' knowledge, this also represents the first description of a live tissue imaging system capable of measuring subcellular motion in *ex vivo* cancer biopsy samples as a biomarker for predicting *in vivo* response to cancer chemotherapy. This significant finding suggests BDI's potential application as a chemosensitivity test for PCM.

Six biomarkers measuring different aspects of subcellular motion were evaluated in this study for their ability to predict the *in vivo* response of canine NHL to doxorubicin chemotherapy. These biomarkers included NSD and DNSD, which reflect aggregate subcellular motion within *ex vivo* tumor biopsy samples, as well as MEM, APOP, BLUETAIL, and FULLSPEC, which reflect discrete types of subcellular motion occurring at restricted anatomic sites within cancer cells. Of these biomarkers, the one demonstrating the greatest ability to predict response to chemotherapy was MEM. A cutoff value of -0.054 for this biomarker showed 100% accuracy for predicting response versus non-response of canine NHL to doxorubicin (area under the ROC curve = 1.00, 95% CI 0.692–1.000). In this population of 10 dogs, high MEM values measured from tumor biopsy samples *ex vivo* were strongly predictive of resistance to doxorubicin chemotherapy *in vivo*. MEM is measured in the middle frequency range (0.1–1.0 Hz) of drug response spectrograms, reflecting motions occurring at the level of the cell membrane [7, 8]. It is possible that these motions may include those produced by membrane-bound drug efflux proteins (e.g. ATP binding cassette proteins). Higher MEM values may in turn reflect increased activity of these proteins which are known to produce chemoresistance. This may explain the strong association between high MEM values and poor clinical response to doxorubicin.

Four other biomarkers, DNSD, APOP, BLUETAIL, and FULLSPEC, also showed good ability to predict the clinical response of canine NHL to doxorubicin (areas under the ROC curve 0.83). A general trend noted in the relationship between these biomarkers and *in vivo* clinical response to doxorubicin was that biomarker values denoting a reduction in cell motion tended to correlate with a positive clinical response (i.e. sensitivity) of a dog's NHL to doxorubicin. Specifically, lower DNSD and FULLSPEC values tended to correlate with a positive clinical response to doxorubicin, as did higher BLUETAIL values. The DNSD biomarker reflects the change in subcellular motion as a whole within tumor biopsy samples following *ex vivo* treatment with doxorubicin. Lower (more negative) DNSD values are indicative of a greater reduction in overall subcellular motion within tumor biopsies. FULLSPEC assesses the change in motion across all frequencies on drug response spectrograms, with lower FULLSPEC values similarly corresponding to an overall reduction

in cell motion. The BLUETAIL biomarker reflects the extent to which motion at any frequency on drug response spectrograms is suppressed following *ex vivo* drug treatment, thus higher BLUETAIL values also correlate with reduced cell motion. Finally, the APOP biomarker reflects patterns of cell motion occurring simultaneously in the high (>1 Hz) and low (<0.1 Hz) frequency ranges on drug response spectrograms. Specific spectral patterns, particularly simultaneous enhancement of motion within both the low and high frequency ranges, have been previously correlated with tumor cell apoptosis [8], a desired outcome of cancer chemotherapy. In this study, lower APOP values tended to correlate with clinical response to doxorubicin chemotherapy.

While the AUC values for several of the motility biomarkers listed in table 2 were high, it should be noted that, with the exception of those for MEM, the ranges of biomarker values for responder and non-responder dogs overlapped (figure 4). Furthermore, the 95% confidence intervals for the areas under the ROC curves for all biomarkers were wide, indicating that precise estimates of each biomarker's ability to accurately classify tumors as responders or non-responders were not afforded by the small sample size in this preliminary study. Further study is therefore indicated to confirm and refine these preliminary data to determine which of these biomarkers, or which combination thereof, has the greatest power to predict *in vivo* clinical response to chemotherapy.

Pet dogs with naturally-occurring NHL were chosen for this work as a relevant animal model in which to test BDI as a predictive chemosensitivity assay. The most compelling rationale for the use of pet dogs with NHL is the biological diversity of these dogs' cancers. Non-Hodgkin's lymphomas in dogs consist of over 30 histopathologically defined subtypes with variable clinical manifestations and responsiveness to chemotherapy [12]. Testing BDI in a population of clinically similar but biologically diverse tumors better recapitulates the inter-patient heterogeneity existing among human cancers than do studies in cultured tumor cell lines or murine tumor xenografts. The response of canine NHL to chemotherapy can vary dramatically, even among dogs with tumors of the same histopathologic subtype. Tumor immunophenotype—whether the tumor derives from B- or T-lymphocytes—is among the strongest predictors of therapeutic response in dogs with NHL. Dogs with T-cell NHL typically experience less robust and less durable responses to doxorubicin-based chemotherapy than dogs with B-cell NHL [20–22]. When treated with single-agent doxorubicin, fewer than 20% of dogs with T-cell NHL will experience CR of their lymphoma. In contrast, over 80% of dogs with B-cell NHL experience CR following doxorubicin treatment [20]. This pattern of therapeutic response was evident in the present study, with 6/8 dogs with DLBCL experiencing CR of their cancer, while neither dog with PTCL-NOS experienced CR. The poor response to doxorubicin chemotherapy in the two dogs with PTCL-NOS could have been predicted to some degree based upon immunophenotype alone. Nevertheless, some of the dynamic motility biomarkers measured by BDI, particularly MEM, showed good-to-excellent ability to predict lack of response to doxorubicin in these two dogs. Perhaps more significantly, the MEM biomarker also accurately identified the 2/8 dogs with B-cell NHL which would fail to exhibit CR to doxorubicin chemotherapy despite belonging to a prognostically favorable subgroup. These observations suggest that BDI may accurately predict failure of a cancer to respond to drug therapy, independent of established histopathologic and clinical prognostic features, and may

therefore be used to identify *a priori* those patients who should be guided towards alternative treatment regimens.

The results presented here provide proof-of-concept to justify further clinical investigation of BDI as a viable method for personalized cancer therapy. However, some limitations to this study should be acknowledged. First, the cancer type under study, NHL, is a highly chemoresponsive hematologic malignancy in both dogs and humans. Biodynamic imaging data from less chemoresponsive solid tumors, which make up the bulk of human malignancies, may be more challenging to interpret and to correlate with clinical response to therapy. Second, considerable heterogeneity in subcellular motion was observed in the processed biopsy samples from the dogs in this study (supplemental figure S4 (stacks.iop.org/CSPO/1/015003/mmedia)). This may be due to inherent biological heterogeneity of the neoplastic cell population comprising individual dogs' tumors. It is well documented that tumors are composed of heterogeneous subpopulations of cells with distinct genomic identities and functional properties [23, 24]. It is likely that these subpopulations exhibit variable subcellular motion profiles as well. However, another possible explanation for the heterogeneity in motion profiles within each tumor sample could be that subcellular motion varied based upon the histologic composition of the tissue (i.e. tumor cells versus stroma) or tissue viability (i.e. live tumor cells versus apoptotic or necrotic cells). We attempted to account for this heterogeneity by averaging the biomarker values from all processed biopsy samples in each dog, then submitting the averaged data to statistical analysis. Further study is needed, though, to determine the optimal number of samples to be analyzed in order to maximize the predictive power of BDI, as well as to better define the mechanisms underlying the heterogeneous motion profiles observed in samples from the same tumor. Because BDI is performed on minimally disrupted living tissue, it could be easily paired with post-hoc assays to determine the extent to which differences in subcellular motion correlate with genomic and proteomic alterations within multiple, anatomically confined regions of a tumor. Furthermore, post-hoc histopathologic evaluation of tumor tissues, which was not part of the design to this preliminary study, would be an invaluable component of future studies to evaluate whether tissue composition is a significant determinant of heterogeneous subcellular motion profiles in biopsy samples from the same patient's tumor.

Conclusion

Biodynamic imaging represents a novel approach to PCM which measures a phenotypic trait—subcellular motion—in *ex vivo* tumor biopsy samples to predict *in vivo* response to drug treatment. This is the first study performed in animals with a naturally-occurring cancer to demonstrate the ability of BDI to predict individual tumors' responsiveness to chemotherapy. In this study, a BDI-measured biomarker related to the motion of cancer cell membranes, MEM, predicted with 100% accuracy the clinical response to doxorubicin chemotherapy in 10 dogs with NHL. Other BDI-measured biomarkers of subcellular motion also demonstrated promise as predictors of clinical response to chemotherapy treatment. These encouraging preliminary results should be substantiated in a larger study population and in the context of multiple or combination drug therapies, which are the standard treatment for most advanced human cancers. Furthermore, trials in which clinical outcomes

are compared in animals treated with BDI-selected therapy and empirically-selected therapy represent a logical next step in validating this technology as a method for personalized cancer treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Characteristic motility contrast images, which depict aggregate subcellular motion averaged over time, of tumor biopsy samples obtained from two dogs with NHL. Images were taken both before and after treating the tumor samples with doxorubicin ($10 \mu\text{M}$). A false color scale bar to the right of the figure characterizes a dynamic motion biomarker (normalized standard deviation (NSD), which describes pixel intensity in the images, averaged over time) for each pixel in the images, with red pixels corresponding to areas of greater aggregate subcellular motion and blue pixels corresponding to areas of lesser aggregate subcellular motion. The two images in the top row depict samples from a non-responder dog's tumor, in which aggregate subcellular motion was unaltered to slightly increased following *ex vivo* doxorubicin treatment. The two images in the bottom row depict samples from a responder dog's tumor, in which aggregate subcellular motion was markedly decreased following *ex vivo* doxorubicin treatment.

Figure 2.

Graphical representation of change in NSD measurement following *ex vivo* application of doxorubicin to tumor biopsies obtained from all 10 study dogs. Mean NSD values (*y*-axis) for the 6 responder dogs (red line) and 4 non-responder dogs (blue line) are plotted as a function of time (*x*-axis). The change in NSD (called 'DNSD') is the net change, evaluated at the end of the measurement time, relative to the baseline.

Figure 3.

Characteristic averaged drug response spectrograms corresponding to tumors from a non-responder dog (A) and a responder dog (B). Frequency of motion detected is plotted on the *x*-axis, and time is plotted on the *y*-axis in each panel. Doxorubicin (10 μ M) was applied at time = 0, denoted by the blue horizontal line near the bottom of each image.

Figure 4.

Dot plots showing the distribution of the dynamic motion biomarker values for the 10 study dogs. Responder dogs are denoted by X's and non-responder dogs are denoted by O's. With the exception of BLUETAIL, in which higher biomarker values tended to correlate with responsiveness to treatment, higher values of most biomarkers tended to correlate with resistance. A cut-off point, which fully discriminates responders from non-responders, is denoted by a red horizontal line on the dot plot for the MEM biomarker.

Table 1

Demographic and treatment response data for dogs with doxorubicin-treated NHL.

Responder dogs						
Dog	Breed	Tumor immunophenotype	WHO subtype	WHO Stage	BOR	PFST (days)
1	Golden retriever	B-cell	N/A	5	CR	154
2	Mixed	B-cell	DLBCL	4	CR	301
3	Mixed	B-cell	DLBCL	4	CR	174
4	Labrador retriever	B-cell	DLBCL	4	CR	180
5	Golden retriever	B-cell	DLBCL	4	CR	210
6	Mixed	B-cell	DLBCL	4	CR	>176

Non-responder dogs						
Dog	Breed	Tumor immunophenotype	WHO subtype	WHO Stage	BOR	PFST (days)
1	Boxer	B-cell	DLBCL	4	PR	37
2	Mixed	B-cell	DLBCL	4	SD	21
3	Mixed	T-cell	PTCL-NOS	5	PD	21
4	Rhodesian ridgeback	T-cell	PTCL-NOS	5	SD	22

Legend. WHO—World Health Organization; DLBCL—diffuse large B-cell lymphoma; PTCL-NOS—peripheral T-cell lymphoma, not otherwise specified; BOR—best overall response to chemotherapy; CR—complete remission; PR—partial remission; SD—stable disease; PD—progressive disease; PFST—progression-free survival time; N/A—not evaluable due to biopsy method (needle core).

Table 2

Areas under the receiver operating characteristic curves (AUC) for the 6 BDI-measured biomarkers, with corresponding 95% confidence intervals (95% CI). From ROC analysis, optimal cutoff values are presented with their associated true positive (TP) and false positive (FP) rates.

Biomarker	AUC	95% CI	Optimal cutoff	TP% (Sens)	FP% (1-Spec)
APOP	0.917	0.555–0.997	−0.072	75%	100%
FULLSPEC	0.875	0.545–0.997	−0.060	100%	83%
MEM	1.000	0.692–1.000	−0.054	100%	100%
BLUETAIL	0.833	0.444–0.975	0.378	83%	100%
DNSD	0.833	0.444–0.975	−0.035	100%	67%
NSD	0.583	0.262–0.878	0.790	50%	83%