Predictive Biomarkers and Personalized Medicine

Comparison of Genomics and Functional Imaging from Canine Sarcomas Treated with Thermoradiotherapy Predicts Therapeutic Response and Identifies Combination Therapeutics

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Abstract

Purpose: While hyperthermia is an effective adjuvant treatment to radiotherapy, we do not completely understand the nature of the response heterogeneity.

Experimental Design: We performed gene expression analysis of 22 spontaneous canine sarcomas before and after the first hyperthermia treatment administered as an adjuvant to radiotherapy. In parallel, diffusion-weighted MRI (DWI) was done prior to the treatment course and at the end of therapy.

Results: From the integrative analysis of gene expression and DWI, we identified significant correlation between tumor responses with genes involved in VEGF signaling, telomerase, DNA repair, and inflammation. The treatment-induced changes in gene expression identified 2 distinct tumor subtypes with significant differences in their gene expression and treatment response, as defined by changes in DWI. The 2 tumor subtypes could also be readily identified by pretreatment gene expression. The tumor subtypes, with stronger expression response and DWI increase, had higher levels of HSP70, POT1, and centrosomal proteins, and lower levels of CD31, vWF, and transferrin. Such differential gene expression between the 2 subtypes was used to interrogate connectivity map and identify linkages to an HSP90 inhibitor, geldanamycin. We further validated the ability of geldanamycin to enhance cell killing of human tumor cells with hyperthermia and radiotherapy in clonogenic assays.

Conclusions: To our knowledge, this is one of the first successful attempts to link changes in gene expression and functional imaging to understand the response heterogeneity and identify compounds enhancing thermoradiotherapy. This study also demonstrates the value of canine tumors to provide information generalizable to human tumors. Clin Cancer Res; 17(8); 2549–60. ©2011 AACR.

Introduction

The clinical benefit of hyperthermia when combined with radiation has been proven in randomized human trials for treatment of melanoma (1), esophageal cancer (2), head and neck cancer (3), cervix cancer (4), and glioblastoma (5). Most recently, neoadjuvant and adjuvant chemotherapy and hyperthermia led to prolonged survival in human soft tissue sarcoma patients compared to chemotherapy alone (6). Despite these successes, the biologic rationale for combining hyperthermia with radiation or drugs is incompletely defined. While heat can kill cells in a time and temperature dependent manner (7), thermal cytotoxicity is not likely to make a major contribution to the clinical effect of hyperthermia due to the inability to uniformly raise temperature to cytotoxic levels (8). Many other effects of hyperthermia on a tumor at the cellular level, other than the heat shock response (9), remain incompletely defined and are likely playing a role in response.

Despite the positive results observed, there are significant variations in the response to hyperthermia treatment between tumors of the same type (10, 11). Understanding how the tumor response to hyperthermia treatment varies may allow the prediction of who will respond to the treatment as well as the potential to identify means to improve the treatment efficacy. One way to achieve these objectives is through the use of microarrays to profile the gene expression of tumors associated with hyperthermia treatment. Although widespread genetic alterations, affecting many important physiologic pathways, have been identified in several studies (12–14), the intratumoral gene expression changes resulting from thermoradiotherapy and...
Translational Relevance

Our work also has the potential to be applied to future practice of cancer medicine. Our research results demonstrate the successful application of both gene expression and functional imaging in determining the nature and heterogeneity in the treatment response in clinical trials. The use of functional imaging in monitoring the tumor physiology and cell killing can be important to provide timely quantitative data on how different tumors respond to cancer therapeutics. The integrative analysis of treatment response can identify critical processes and candidate biomarkers genes associated with the varying degrees of treatment response to predict and individualize the treatment plan. The differential gene expression between tumors with distinct response can also be used to identify compounds which will enhance the cancer therapeutics. Therefore, our study has potential to impact the future practice of clinical trials and cancer medicine. This manuscript also complies with the criteria of original research with a clear application to future practice of medicine. This study is also hypothesis testing, possesses robust statistics, and is biologically based. The gene expression result from canine sarcoma is further tested in human cells. We believe this work will be of considerable interest to readers of Clinical Cancer Research.

their relationship with tumor response are not well understood. Such definition of gene expression can also be analyzed with bioinformatics tools and other existing gene signatures representing specific biological pathways and processes. The simultaneous characterization of gene expression in concert with other biologic endpoints can uncover relationships to better understand how these changes come about as well as identify additional therapeutic targets. Most importantly, these methods can allow for unexpected connection in gene expression to provide mechanistic insights into response heterogeneity and identify means to further improve therapeutic benefit.

Our purpose in this study was to assess gene changes occurring in canine spontaneous soft tissue sarcomas as a result of thermoradiotherapy. In addition, we compared gene changes to changes in tumor volume and the apparent diffusion coefficient (ADC) of water, quantified using diffusion-weighted MRI (DWI). We applied this analysis to spontaneous soft tissue sarcomas since they are more similar than rodent tumors to human cancers (15) in terms of intertumoral heterogeneity, multiple mutations in oncogenes and tumor suppressor genes, and varying environmental conditions. Through such integrative analysis, we identified many important genes which are strongly correlated with treatment response. The variation in gene expression response also led to identification of 2 tumor subtypes characterized by a significant difference in the effect of treatment on the ADC. Analysis of differential gene expression among these 2 subtypes allowed us to identify genes associated with them and to identify compounds which may enhance the therapeutic efficacy of hyperthermia. We further validated the ability of one identified compound, geldanamycin, to enhance the cell killing of hyperthermia and radiotherapy in clonogenic assays.

Materials and Methods

Design and subjects
Twenty-two dogs with a spontaneous soft tissue sarcoma were studied. Tumor types included hemangiopericytoma (HPC, n = 12), undifferentiated sarcoma (Sarcoma, n = 6), fibrosarcoma (FSA, n = 3), and peripheral nerve sheath tumor (PNST, n = 1). Canine hemangiopericytoma is a malignant mesenchymal tumor with a suspected pericyte origin (16). It behaves similar to other malignant mesenchymal tumors in the dog, being locally invasive and having metastatic potential associated with histologic grade.

Tumors were graded histologically as either low or high grade based on the number of mitoses per high power field; there were 5 high grade tumors, 16 low grade tumors, and 1 tumor of unknown grade. Fifteen tumors were located on an extremity, 5 on the trunk, and 2 on the head. No tumor was judged to be resectable completely, without amputation, prior to treatment. Median tumor volume, measured physically with calipers and computed as the product of 3 orthogonal diameters divided by π/6, was 80 cm³, with a range of 17 to 180 cm³.

Dogs were part of an institutionally-approved randomized trial investigating the effect of thermal dose fractionation when combined with radiotherapy. This trial involved a 5-week treatment of daily fractionated radiation therapy and 1 of 2 hyperthermia prescriptions. Prescription 1 was 1 hyperthermia treatment per week and prescription 2 was approximately 3 hyperthermia treatments per week. The total thermal dose was the same in each prescription.

Dogs received local hyperthermia under anesthesia using a superficial 433 MHz microwave applicator. The hyperthermia technique has been described elsewhere in detail (17). The radiotherapy prescription was 56.25 Gy administered to the planning target volume in 25 daily fractions of 2.25 Gy using 6 MV photons. Dogs received either 1 or 2 radiation fractions prior to the posttreatment biopsy (Supplementary Fig. S3).

Outcome variables
The percent change in tumor volume at the end of the 5-week treatment was used as the measure of tumor response. For economic reasons it was not possible to longitudinally assess animals for tumor regrowth following completion of therapy. Most tumors remained unrectactable after therapy, making histologic assessment of the entire tumor also impossible.

Water diffusion was quantified in 16 of the 22 dogs prior to treatment and at the end of treatment using DWI with a 1.5 T unit (Siemens Symphony, Siemens Medical Systems).
DWI was performed using a half Fourier acquisition, diffusion-weighted, single shot turbo spin echo (HASTE) sequence with \( b \) values of 0 and 500 sec/mm\(^2\) (TR = 3,000 msec, TE = 132 msec, echo train length = 256, 128 × 128 matrix, NEX = 2 for \( b = 0 \) sec/mm\(^2\) and NEX = 6 for \( b = 500 \) sec/mm\(^2\), 4.0-mm slice thickness, 0.5 mm gap). Diffusion-weighted images were used to compute the apparent diffusion coefficient (ADC) using proprietary software provided by the manufacturer. T2-weighted spin echo images were also acquired and used for anatomic registration of the ROI used for quantification of ADC. The ADC was quantified in every pixel in the tumor by drawing a region of interest around the tumor in every ADC image. ADC was quantified using ImageJ (http://www.rsb.info.nih.gov/ij/). The change in ADC was expressed as the percent change in mean ADC at the end of treatment compared to the mean pretreatment ADC value. ADC images and a histogram depiction of pretreatment and posttreatment pixel values are shown in Supplementary Figures S1 and S2.

**Gene expression analysis**

Tumor biopsies for gene expression analysis were obtained prior to any treatment and 24 hours after the first hyperthermia treatment. Tumor biopsies were obtained with an 18 g Tru-Cut needle and inspected grossly for quality. Multiple samples were collected with one assessed for gene expression; the others were used for other endpoints being described elsewhere. Some of the other biopsy samples were stained with hematoxylin and eosin stain (H&E) and verified that viable tumor was obtained. Tumor biopsies were obtained with an 18 g Tru-Cut needle and inspected grossly for quality. Multiple samples were collected with one assessed for gene expression; the others were used for other endpoints being described elsewhere. Some of the other biopsy samples were stained with hematoxylin and eosin stain (H&E) and verified that viable tumor was obtained.

There was some variation in time between the pretreatment biopsy and the first treatment that was caused by scheduling conflicts. The median time between the pretreatment biopsy and the first treatment was 84 hours. Dogs received either one (\( n = 12 \)) or two (\( n = 10 \)) 2.25 Gy radiation fractions prior to the posttreatment biopsy. The posttreatment biopsy was always obtained 24 hours after the first heat treatment. The timing of all sample collections, treatments, and endpoint evaluations is indicated in Supplementary Figure S3.

Tissue used for gene expression analysis was placed immediately into RNA Later for RNA harvesting with Qiagen RNAeasy and quality was checked by an Agilent Bioanalyzer. RNA was labeled with Affymetrix One-Cycle Labeling Reagents and then hybridized to Affymetrix Canine 2.0 GeneChips® to measure the expression of 18,000 *C. familiaris* mRNA/EST-based transcripts and over 20,000 nonredundant predicted genes.

**Statistical analysis of gene expression data**

Gene expression profiles of the 44 biopsies from the 22 tumors (Gene Expression Omnibus, GEO, accession number: GSE23380) were normalized by the Robust Multichip Average (RMA) algorithm (18), filtered by indicated criteria and arranged by hierarchical clustering (Gene Cluster program and 2-way average linkage clustering based on uncentered correlation; ref. 19). For each probe set, the change in expression was quantified by subtracting the expression value of the pretreatment from the expression value of the corresponding posttreatment samples. Given that the RMA algorithm provides log (base 2) transformed summary measures, this difference corresponds to the fold of changes caused by treatments. The binary regression prediction model was used to build gene signature predictive model (2 metagenes, 150 probsets) with Matlab from the pretreatment samples. Briefly, a signature represents a group of genes that collectively demonstrate a consistent pattern of expression (Supplementary Fig. S4A) in relation to the pretreatment tumor grouping. The gene selection, identification, and regression model is based solely on the training data (pretreatment tumors) and maintains statistical independence from the validation dataset (posttreatment tumors). The predicted probabilities in the leave-one-out validation (pretreatment; Supplementary Fig. S4B) and independent validation (posttreatment; Supplementary Fig. S4C) were shown.

To test whether the physiologic continuous outcomes varied between 2 groups, the 2 sample Wilcoxon rank sum test (20) was used. To identify probe sets expressed differentially, the Wilcoxon signed rank test was used. To test whether the change in expression is associated with continuous outcomes (e.g., tumor volume, ADC), the Spearman rank correlation test was used. The significance of the identified association was further verified by permutations (1,000).

SAM (21) and PAM (22) were used to identify genes associated with treatment or subgroups. The selected genes were then merged with the GoodMatch list provided by Affymetrix to convert the corresponding human probe sets for gene ontology (GO) enrichment analysis in gather (23) and connectivity map (24). One thousand, five hundred and eighty one probe sets were identified by SAM between the 2 tumor groups with a false discovery rate (FDR) of 0.16% (Fig. 6A). These probe sets were merged with GoodMatch list from Affymetrix website using filemerger (http://filemerger.genome.duke.edu/) to the corresponding human probe sets in U133A2 for analysis using connectivity map (ref. 24; http://www.broadinstitute.org/cmap/).

For the PAM selection of the genes separating the 2 tumor subtypes, a threshold of 3 (Supplementary Fig. S7A) was selected to yield the cross-validation error rate of 18% (Supplementary Fig. S7B).

**SYBR green based qPCR**

The NCBI Dog Genome Resource was used to identify the mRNA sequence of genes of interest for primers designed by primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). One microgram of total RNA was reverse transcribed into cDNA using a Qiagen Quantitect Reverse Transcription Kit (Qiagen, Inc.). Fifty nanogram of cDNA was used as a template for qPCR. In addition to the template cDNA, the qPCR reaction contained forward and reverse primers, each at a concentration of 300 nmol/L, and 1 × SYBR green I fluorescent dye, per manufacturers instructions (Power SYBR Green PCR Master Mix, Life
Technologies Corp.). qPCR was performed with an ABI StepOne real-time PCR machine (Life Technologies Corp.) with thermal cycling conditions of 15 minutes at 95°C and 40 cycles of 15 seconds at 95°C plus 1 minute at 60°C. Relative quantification was determined using the Pfaffl method standardized to the gene for canine ribosomal protein RPLP0, accession number XM_853158.1 (F: CAGCAATGGAAAGTGTTAATCAG, R: CCCATCTCATCATAAGATAAC).

PECAM1: F: GCCAGCCATCTGGCCTGGGATT, R: ACTGCCCTTGCGTCG.

WVF: CCCAGTGCTCCCAGAAGCCCT, R: TCGCCCGTGGTGAACCG.

ENO II: F: GATACAGCCCCTCCTGCTCTCCC, R: TCCACCACCGCCTCCTCCT.

Transferrin: F: TGACCAGCAGCTTTGTTTG, R: ACATGTGTGCCTCCTGTA.

BBX: F: TACCAACCCAGCTATCAGCA, R: GCACCCCTTGAGGCTC.

HSP70: F: ATCACCCATCAACACGACAA, R: GAAGCCCTAGGACTCCAG.

Clonogenic survival assays

HCT116 cells were plated in 6-well plates at 2 different densities (300 and 900 cells per well) in 3 mL of DMEM media and allowed to attach for 24 hour at 37°C. Geldanamycin (5 or 10 nmol/L) or the vehicle (DMSO) was added to the cells for 1 hour at 37°C. Then the plates were wrapped with foil before being placed in temperature-controlled water baths for heat treatments at either 37°C or 42°C for 1 hour. For the radiation treatments, cells received 2 Gy using a Cesium-137 Irradiator immediately post heating. The media containing drugs was removed 6 hours after radiation or hyperthermia treatment and the cells incubated in fresh media at 5% CO₂ and 37°C for 7 to 10 days. After incubation, cell colonies were fixed with 10% methanol–10% acetic acid and stained with a 0.4% crystal violet solution. Colonies with more than 50 cells were counted using a ColCounter (Oxford Optronix). Plating efficiencies were determined for each treatment and normalized to the vehicle controls. The data shown is the mean of 3 independent experiments.

Results

The functional imaging studies of treatment response

We measured tumor volume prior to and at the end of the treatment using DWI. Significant variation in the percent ADC change was observed between tumors. ADC decreased in 8 of 16 dogs and increased in 8 of 16 dogs at the 5-week measurement point compared to the pretreatment measurement. The range of change was from −49.9% to +68.9% (Fig. 1A). Tumor volume decreased in 21 of 22 dogs and increased slightly in 1 of 22 dogs at the end of treatment compared to the pretreatment tumor volume. The range of volume change was from −94.3% to +3.6% (Fig. 1B). There was a statistically significant association between the percent change in tumor volume and the percent change in ADC (Fig. 1C).

The gene expression studies of treatment response

Tumor biopsies were obtained from all 22 subjects prior to any treatment and 24 hours after the first hyperthermia treatment. Gene expression profiles of the 44 samples were normalized by RMA and deposited into GEO (accession number: GSE23380). To reduce the confounding factor variation between different of individual tumors, we used zero-transformation to characterize treatment-induced gene expression by comparing gene expression for the same tumors before and after treatment. This analytic approach, which has been used successfully in several studies (25–29), involves the subtraction of the pretreatment expression value of a probe set from the posttreatment expression value of the same probe set. From the treatment-responsive gene expression, we selected 2712 probe sets having at least a 2-fold change in at least 2 samples and arranged by hierarchical clustering (Fig. 2).

When all 22 samples were grouped using hierarchical clustering in an unsupervised analysis based on changes in gene expression in response to thermoradiotherapy, there were 2 distinct groups of tumors with 15 tumors in 1 group (group I, marked by a red bar in Fig. 2A) and the remaining 7 tumors in the second group (group II, marked by a blue bar in Fig. 2A). The separation of these 2 groups of tumors was mostly due to several large gene clusters which were only induced in group I tumors (Fig. 2A and B) and was robust by various filtering criteria. The group I tumors exhibited a much stronger gene expression response as manifested by larger number of genes which were induced only in the group I tumors. The separation of these 22 tumors was not due to histopathologic types or treatment regimes (Fig. 2A). Importantly, the clustering pattern of gene expression changes after thermoradiotherapy was significantly (P = 0.0275) associated with varying amount of changes in ADC at the end of treatment, 5 weeks later (Fig. 2C).

Large clusters of genes were induced and repressed in response to thermoradiotherapy (Fig. 2A and B). These treatment-altered genes can be classified as either altered in most tumors (common response) or seen in only a subgroup of tumors (group 1 specific response). Commonly induced genes included many genes in the heat-responsive (heat-responsive protein 12), DNA damage response (Mdm2), and cell cycle inhibition (p21/Cip; Fig. 2B). Genes commonly repressed by thermoradiotherapy included genes (Fig. 2B) involved in cell cycle progression, confirming a reduction in cellular proliferation in response to treatment. Cell cycle inhibition is known to be associated with both radiation and hyperthermia responses. Hyperthermia has been shown to induce both G1 and G2 cell cycle delays (30, 31), whereas the major cell cycle delay seen after radiation treatment is a G2 delay (31).
Since these 2 tumor subtypes were identified based on their differential gene expression response to treatments, we explored the possibility to predict their response based solely on pretreatment gene expression. We used the binary regression model to build a gene signature predictive model based on the differential expression of 150 genes among the expression of tumors in the group I vs. II before treatment (Supplementary Fig. S4A). In the leave-one-out validation experiments, we found that this model successfully predicted most of the tumor grouping (accuracy: 95%, Supplementary Fig. S4B), revealing the consistency of the grouping and validity of the prediction models. We also used the posttreatment samples, not used in the building of the predictive models, to further validate the prediction models. By using a probability threshold of 0.6, the model was able to classify the tumors as group I vs. II with accuracy of 82% (Supplementary Fig. S4C). If the probability threshold was set at 0.5, the predictive accuracy dropped to 72%. These results suggest the possibility to predict the treatment response based on the gene expression prior to treatment for individual tumors.

The tumor heterogeneity revealed by gene expression response

There was a large group of genes which were only induced in group I tumors (Fig. 2B). These genes encode proteins involved in tissue remodeling (e.g., MMP-1, MMP-2A, MMP-3, TGF-β, CD44, SERPINA1, SEPINB6, and SRF) and inflammatory and immune responses [CCR1, interleukin 1β (IL1β), IL6, IL8, IL10, MARCO]. The changes in gene expression of tissue remodeling are entirely consistent with the higher changes in ADC in this group (Fig. 2C). These results demonstrate our ability to use microarrays to elucidate the biological effects and mechanistic insights into the response and heterogeneity of the hyperthermia treatment.

Integrative analysis of gene expression and functional imaging

The quantitative measurements of the response of tumor volume and ADC by functional imaging allowed us to identify genes whose expression response was associated with these parameters on a linear and continuous scale (Supplementary Table S1). Among the genes selected to be
We also identified several genes with biological interest. For example, the expression response of BRCA1 was significantly negatively associated with the change in ADC (Fig. 3A). In addition, the expression response of Flt1 and KDR, which encodes 2 receptors for VEGF, were strongly positively associated with changes in ADC (Fig. 3B and C). Increased expression of Flt1 and KDR 24 hours after the first hyperthermia treatment were also associated with an increase in ADC at 5 weeks. We also identify a strong positive correlation between the changes in ADC with both Stat5 (encodes a mediator of inflammatory response) and ACCN3 (encodes an acid-sensing channel protein). To further verify the statistical significance, we recalculated the $P$ values for these probe sets with 1,000 sample permutations (Supplementary Table S3).

We also applied similar analysis to the response of tumor volume (Fig. 4). We identified strong positive association between the percent change in tumor volume at the end of therapy and expression of telomerase reverse transcriptase (TERT). Tumors with reduced expression of TERT following the first hyperthermia treatment had greater decreases in tumor volume at the end of therapy (Fig. 4A) which is consistent with the known role of the telomerase in tumor growth. We also found an inverse relationship between tumor volume response and expression of RAD23A and BRIP1 (BRCA1 interacting protein C-terminal helicase 1; Fig. 4B and C), 2 genes encoding proteins important for DNA damage repair. RAD23A encodes a human homologue of Saccharomyces cerevisiae Rad23, a protein involved in nucleotide excision repair (NER). BRIP1 encodes a protein member of the RecQ DEAH helicase family and interacts with the BRCT repeats of BRCA1 in a protein complex which is important in the normal double-strand break repair function of BRCA1. We have also noted a significant positive correlation of tumor volume with HMGCR (3-hydroxy-3-methylglutaryl-coenzyme A reductase), RPS11 (Ribosomal protein S11; Fig. 4D and E), and EEFSEC (eukaryotic elongation factor, selenocysteine-tRNA-specific). To further verify the statistical significance, we recalculated the $P$ values for these probe sets with 1,000 sample permutations (Supplementary Table S3).

**Supervised analysis of genes and compounds associated with thermoradiotherapy**

To identify the genes whose expression was consistently associated with untreated and treated tumors, we used a supervised method, Prediction Analysis of Microarray (PAM)
(PAM) to prioritize the differentially expressed mRNAs from array analysis (22). PAM is a class prediction tool based on the shrunken centroids of gene expression, which computes a standardized centroid for each class and selects the number of genes required to characterize each assigned class with a defined error rate. PAM analysis showed that only 35 probes were needed to achieve 100% accuracy of prediction of treatment effects. The change in the gene expression of these 35 probes (Supplementary Fig. S5) showed the downregulation of topoisomerase and RAD23 and the upregulation of mdm2 and p21 in both groups. Among the top genes induced in the treated samples are the Cyclin-dependent kinase inhibitor 1A (p21, Cip1) and MDM2. Our PAM analysis was consistent with these processes of cell cycle regulation as well-known consequences of heat and radiation (30). Taken together, these results suggest that a prominent and consistent effect of thermoradiotherapy is the upregulation of regulators of cell cycle progression and the resulting arrest of cell cycle.

Supervised analysis of gene expression and compounds associated with the 2 tumor subtypes

We also used PAM to prioritize the differentially expressed mRNAs associated with the 2 tumor subgroups identified by varying gene expression responses and found that only 110 probes were needed to achieve more than 90% accuracy of prediction (Fig. 5 and Supplementary Fig. S6). The level of gene expression of these 110 probes among these 2 subgroups is shown in Figure 5A. The differential expression of the hsp70, TOP1, CD31, and VWF among these 2 tumor subtypes was further tested statistically in nonpaired t-test and found to be significant (Fig. 5B and C). The tumors in group I had a consistently higher expression level of hsp70, POT1 [protection of telomeres 1 homologue (S. pombe)], CDC23 [a component of anaphase-promoting complex (APC)] and several genes comprising the centrosome (centrin, centrosomal protein 70 and 192 kDa). The higher level expression of hsp70, BBX, and ENOII in group I tumor was verified by RT-PCR (Supplementary Fig. S7). Higher level of hsp70 may contribute to the exaggerated response to the hyperthermia treatments, consistent with previous reports using immunohistochemical staining (32).

In contrast, in group II tumors there was a higher expression level of many genes known to be expressed in mature blood vessels, including endothelial and smooth cells. These genes include CD31, VWF, melanoma cell adhesion molecule (MCAM), myosin, heavy chain 11,
smooth muscle, and myosin light chain kinase (Fig. 5A and C). The higher mRNA expression level of transferrin, CD31, and vWF in group II tumors was also verified by real-time RT-PCR (Supplementary Fig. S7). The high level of endothelial and smooth muscle-specific genes may suggest a higher level of mature vasculature and could contribute to the modest gene expression response to thermoradiotherapy. This may limit the temperatures achieved during heat treatment and/or reduce the gene expression to the applied heat stresses, consistent with a previous report (33).

Identification of compounds which enhance the efficacy of hyperthermia

We then investigated the potential of using the differential gene expression among the 2 groups and connectivity map to identify compounds with the potential to enhance the therapeutic efficacy of hyperthermia treatment. We used Significant Analysis of Microarrays (SAM; ref. 21) to identify probe sets whose expression varied consistently between the 2 clusters (Fig. 6A). These differentially expressed probe sets were used to interrogate the connectivity map (24) to establish connection with compounds inducing similar or opposite expression. These compounds included TSA and MS-275, both known to be inhibitors of histone deacetylase (HDAC; Supplementary Table S5). In addition, geldanamycin, a known inhibitor of hsp90, was also found to be significantly enriched (Supplementary Table S5). These compounds are likely to affect how cells respond to the hyperthermia because their ability to impact the heat shock proteins and heat shock response.

To test the ability of compounds identified to synergize with heat treatment, we performed clonogenic survival assays using HTC-116 colon carcinoma cells treated at 37°C for 30 and 60 minutes in the presence of different concentrations of geldanamycin (GA). Radiation was administered immediately after hyperthermia treatment was completed. Geldanamycin was removed 6 hour after heat treatment. Clonogenic assays of human HCT116 colorectal carcinoma cells pretreated 1 hour with the indicated concentrations of geldanamycin, then exposed to either 37°C or 42°C for 1 hour alone (Fig. 6B) or immediately followed by 2 Gy radiation (Fig. 6B and C). Analysis of survival data indicated that geldanamycin significantly enhanced cytotoxicity of hyperthermia and radiation alone as well as hyperthermia combined with radiation ($P < 0.0001$; Fig. 6B and C). Collectively, these results demonstrate that analysis of differential gene expression between...
canine tumors with different treatment response allowed the identification of compounds which enhanced the therapeutic efficacy of thermoradiotherapy of human cells.

Discussion

The integrative analysis of gene expression and functional imaging

In this study, we performed an integrative analysis of both gene expression and functional imaging associated with thermoradiotherapy of spontaneous canine sarcomas. The functional imaging of tumors with DWI provided quantitative analysis of changes in the tumor related to water content and cell density, which may be related to treatment outcome. The association we found between reduction in ADC and reduction in tumor volume supports the association of ADC and treatment outcome (Fig. 1C). Interestingly, most solid tumor studies where ADC has been assessed as a predictor of outcome associate increases in ADC with better outcome (34). In this study, we found the opposite, assuming that reduction in volume is a sign of better outcome. However, there are no studies of changes in ADC during therapeutic hyperthermia where tumor control duration has been quantified therefore, whether increases or decreases in ADC as a response of tumor heating signify improved response is not known. Some data regarding ADC change are available from thermal ablation studies (35, 36) and in that setting a decreased ADC was associated with better tumor response. The temperatures used in the hyperthermia treatments in this study were lower than those used for thermal ablation but perhaps there is another effect of heating on the tumor that leads to reduced water diffusion in favorably responding tumors. More work is needed on the association between ADC and outcome in tumors undergoing hyperthermia.

Imaging data, coupled with gene expression analysis allowed us to link sets of genes associated with tumor physiologic function. Further, gene expression responses revealed subtypes of tumors exhibiting varying degrees of treatment response. The differences in ADC between groups I and II also demonstrate the potential clinical relevance of these 2 subtypes revealed by gene expression analysis. Thus, the availability and reciprocal flow between these 2 levels of quantitative information offers unique advantages in understanding how physiologic changes in tumors after thermoradiotherapy are linked to gene expression changes. The differential gene expression among these 2 subtypes both enables the development of gene expression predictive of putative subtypes and discovers compounds which enhance the therapeutic efficacy of thermoradiotherapy.

The identification of genes associated with tumor response from such integrative analysis also has the potential to provide biological insights into treatment response. For example, the changes in expression of 2 VEGF receptors, Flt1 and KDR, with treatment response suggested an association between VEGF signaling and effects of thermoradiotherapy treatment on solute transport. The results are consistent with our preclinical data showing that both...
radiation and hyperthermia increase HIF-1 levels, which drives VEGF signaling (37, 38). In addition, one would expect that water diffusion will increase in response to more cell killing (39). Thus, the change in ADC most likely reflects both types of effects. The association of Stat 5 with ADC response is also of interest since Stat5 encodes a protein that serves the dual function as a signal transducer and activator of transcription in cells exposed to many inflammatory cytokines. Such positive correlation was consistent with the induction of several inflammatory genes (Fig. 2B) in the subgroup I tumors with a higher response in ADC (Fig. 2C). The association of tumor volume response with genes involved in various biological processes suggests a rationale for combining many agents targeting HMGCR, telomerase (40) and VEGF with hyperthermia.

The advantages of studying spontaneous canine sarcoma

Spontaneous canine soft tissue sarcomas exhibit intertumoral heterogeneity due to multiple mutations in oncogenes and tumor suppressor genes, varying environmental conditions, and inherited germline variations (15). The combination of these factors leads to immense natural heterogeneity in tumor phenotypes, disease outcomes, and response to therapies. This is certainly the case for the varying response to hyperthermia treatment. We are not stating that the canine sarcoma is an identical model of human sarcoma, only that it is a spontaneous tumor characterized by many of the same heterogeneities that characterize various human tumors. And, the physics of hyperthermia treatment, although not discussed in detail here, is identical to that of human tumor thermal therapy adding more validity to the model.

The advent of DNA microarray technology allows the study of gene activity and gene function at a genome level as a means to analyze the molecular phenotypes and clinical heterogeneity of tumors. Such analysis in this cohort of canine sarcomas revealed that the gene expression of tumors is affected significantly, but not uniformly, by thermoradiotherapy. While certain genes are affected in most tumors, the majority of the affected genes are found only in 1 subgroup of the tumors. Such heterogeneity revealed from the parallel analysis of functional imaging and genomic profiling may also be extended and generalized to the hyperthermia treatment of human cancers. This potential is supported by the ability of compounds identified from the gene expression of canine tumors to enhance the thermoradiotherapy-induced killing in human cancer cell line.

Common and subgroup-specific response genes affected by thermoradiotherapy

Among the genes affected by thermoradiotherapy in most tumors, we have found the induction of MDM2, p21, and the reduction in the genes involved in cellular proliferation. The induction of these genes implicates the p53 as important regulator in the thermoradiotherapy response, consistent with the previous reports on the important role of p53 in hyperthermia and thermoradiotherapy treatment responses (41–45). We also
examined genes affected in only the subgroup I of the tumors as a means to predict and improve the treatment response. The compounds identified in the connectivity map through the differential gene expression among subtypes may also lead to mechanistic insights into thermoradiotherapy response. This connection with the hsp90 inhibitors suggests their role in determining tumor response to thermoradiotherapy. Hsp90 is an important chaperone responsible for the stability of many proteins involved in oncogenesis (46). Under the hyperthermia treatments, Hsp90 is likely to play an even bigger role in the survival and growth in response to heat and oxidative stresses caused by the treatments. In addition, hsp90 is also functionally regulated by acetylation by HDAC6/HDAC4. Thus, our gene expression analysis has suggested the critical role of the HDAC-hsp90 pathway for the adaptive response under the stresses of thermoradiotherapies. Given the large number of compounds that are under development to target HSP90; these agents are likely to further enhance the therapeutic benefits of thermoradiotherapies.

The potential of gene expression to predict tumor response to thermoradiotherapy

Here, we found that gene expression of tumors before treatment can be used to build a gene expression model predictive of the likelihood for response to thermoradiotherapy. These selected genes can be further validated for their predictive power in independent cohorts of tumors undergoing thermoradiotherapy. Based on the analysis of gene expression data, we found that tumors with high expression levels of hsp70 and centrosomal proteins are likely to have stronger response to thermoradiotherapy. Our findings suggest that changes in hsp70 level in tumor after hyperthermia may be useful to predict tumor response. This result is consistent with previous studies of the ability of hsp70 to predict hyperthermia response (32) and clinical outcomes (47). It is also interesting to note there is a higher expression level of endotelial and smooth muscle associated genes expressed in the subgroup II with less ADC response. This link of tumor blood vessel maturity with hyperthermia treatment response has previously been recognized (48) and suggests the value of combination treatment with many antiangiogenesis treatments.

Comparison with other gene expression studies of hyperthermia

Gene expression analysis has been previously used to analyze hyperthermia treatment response in several different contexts (12–14, 49, 50). Our findings of the relevance of the DNA damage cell cycle (14) and angiogenesis pathways (13) are consistent with several other studies. Previous studies also point to the changes in the inflammatory pathways associated with thermoradiotherapy (12, 50), as seen in our subgroup-specific gene expression response. However, our studies also differ from the previous studies since we used functional imaging to directly measure the pathophysiologic response to treatment. In addition, we have also used heterogeneity in response to develop predictive gene signatures and identify geldanamycin enhancing the cell killing by thermoradiotherapy. Such use of gene expression may allow the codevelopment of both synergistic therapeutics and predictive biomarkers to identify patients who are likely to benefit from such therapeutics. Taken together, the gene expression studies of hyperthermia-treated spontaneous canine tumors shed mechanistic insights into the heterogeneity of thermoradiotherapy response. To our knowledge, this is one of the first successful attempts to link genomic and functional imaging changes together and use such associations to understand the connection between cellular and physiologic responses to treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


Chi et al.

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