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Feasibility of using gene expression analysis to study canine soft tissue sarcomas

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Abstract The prognosis given for canine soft tissue sarcomas (STSs) is based primarily on histopathologic grade. The decision to administer adjuvant chemotherapy is difficult since less than half of patients with high-grade STSs develop metastatic disease. We hypothesize that there is a gene signature that will improve our ability to predict development of metastatic disease in STS patients. The objective of this study was to determine the feasibility of using cDNA microarray and quantitative real-time PCR (qRT-PCR) analysis to determine gene expression patterns in metastatic versus nonmetastatic canine STSs, given the inherent heterogeneity of this group of tumors. Five STSs from dogs with metastatic disease were evaluated in comparison to eight STSs from dogs without metastasis. Tumor RNA was extracted, processed, and labeled for application to the Affymetrix Canine Genechip 2.0 Array. Array fluorescence was normalized using D-Chip software and data analysis was performed with JMP/Genomics. Differential gene expression was validated using qRT-PCR. Over 200 genes were differentially expressed at a false discovery rate of 5%. Differential gene expression was validated for five genes upregulated in metastatic tumors. Quantitative RT-PCR confirmed increased relative expression of all five genes of interest in the metastatic STSs. Our results demonstrate that microarray and qRT-PCR are feasible methods for comparing gene signatures in canine STSs. Further evaluation of the differences between gene expression in metastatic STSs and in nonmetastatic STSs is likely to identify genes that are important in the development of metastatic disease and improve our ability to prognosticate for individual patients.

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Introduction

Soft tissue sarcomas (STS) are common but varied skin and subcutaneous tumors in dogs, comprising many different tissues of origin. These tumors are locally invasive, and recurrence is common following surgical excision. The metastatic potential of individual STSs is difficult to predict but currently is based primarily on histologic grade. Two grading schemes have been proposed to predict metastasis. The grading scheme proposed by Bostock and Dye (1980) differentiated low-grade from high-grade tumors based on mitotic index only, while a later scheme proposed by Kuntz et al. (1997) defined low-, intermediate-, and high-grade tumors based on mitotic index, tumor necrosis, and degree of differentiation.

The overall metastatic rate of canine STSs has been described as 13% for low-grade tumors, 7% for intermediate-grade tumors, and 41% for high-grade tumors (Kuntz et al. 1997). However, a study by Heller et al. (2005) identified a metastatic rate of 29.5% for low-grade tumors and 34.6% for high-grade tumors, demonstrating the difficulty of applying grading schemes to predict metastasis in STS. Therefore, additional methods have been evaluated for predicting tumor behavior. These have included cellular proliferation markers such as argyrophilic nucleolar organizing regions (AgNORs), Ki-67, and proliferating cell nuclear antigen (PCNA) scores, as well as evaluation of

tumor microvessel density (Ettinger et al. 2006; Luong et al. 2006). Increased AgNOR and possibly Ki-67 expression were associated with decreased survival, while PCNA expression had no association with survival (Ettinger et al. 2006). Increased intratumoral microvessel density was associated with increasing grade, mitotic index, and metastatic potential (Luong et al. 2006).

The decision to administer adjuvant chemotherapy to a canine STS patient can be difficult, since less than 50% of sarcomas will metastasize despite tumor grade. The most commonly administered adjuvant chemotherapy for STS is doxorubicin (Ogilvie et al. 1989; Tilmant et al. 1986). In the only published report on the efficacy of doxorubicin in a small number of dogs with high-grade STS, no benefit was found in preventing the development of metastatic disease (Selting et al. 2005). Doxorubicin has also been associated with significant side effects; in addition to bone marrow suppression and gastrointestinal toxicity, cumulative cardiotoxicity has been reported in 12–22% of dogs (Gillings et al. 2009; Mauldin et al. 1992; Page et al. 1992).

Recent studies in human medicine have analyzed gene expression signatures in various tumor types, including STSs (Baird et al. 2005; Landemaine et al. 2008; Nilbert et al. 2004; Ramaswamy et al. 2003). Unique gene expression patterns have been used to delineate specific tumor types or to predict tumor metastasis. The objective of this study was to determine if gene expression analysis using a cDNA microarray and quantitative real-time PCR (qRT-PCR) is a feasible approach for differentiating metastatic and nonmetastatic STSs in dogs, given the inherent heterogeneity in these tumors. We hypothesized that there is a unique gene expression pattern that can predict an STS's likelihood of metastasis. The ultimate goal is to identify a gene signature that is unique for metastatic sarcomas and to use this information to screen patient tumor samples and more accurately determine the need for adjuvant chemotherapy.

Materials and methods

Tumor samples

Samples from 13 primary canine STS tumors (five metastatic, eight nonmetastatic) were stabilized at room temperature in RNAlater® (Ambion, Inc., Austin, TX) and then stored at −80°C. Samples in the nonmetastatic group were from patients free of metastatic disease at least 1 year from the time of diagnosis.

RNA extraction

Approximately 30 mg of tumor tissue was used for RNA extraction, which was performed using the Qiagen RNeasy MiniKit (Qiagen Sciences, Germantown, MD). Samples were lysed and homogenized using Qbiogene ceramic spheres (Qbiogene/MP Biomedicals, Irvine, CA) and a bead beater (BioSpec Products, Bartlesville, OK). RNA quantity and integrity were assessed with the NanoDrop (Thermo Scientific, Wilmington, DE), as well as the Agilent Technologies RNA NanoChip on the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc, Santa Clara, CA). Only RNA samples with an RNA Integrity Number (RIN) of seven or greater were used for labeling and hybridization to the microarray.

Labeling and hybridization

The Affymetrix One-Cycle Target Labeling Kit (Affymetrix, Inc., Santa Clara, CA) was used to convert RNA to cDNA and then to label cRNA. cRNA integrity was reassessed using the RNA NanoChip on the Agilent 2100 Bioanalyzer, and again, only cRNA with a RIN $\geq$ 7 was used. Sample cRNA was hybridized to the Affymetrix Canine Genechip® 2.0 Array (Affymetrix) at the Duke University Microarray Facility in Durham, NC.

Microarray data analysis

Microarray fluorescence was normalized using dChip software (Li and Wong 2001). Data analysis was performed using JMP/Genomics software (SAS Institute, Cary, NC) to identify genes differentially expressed between metastatic and nonmetastatic tumors at a false discovery rate of 5% using ANOVA.

Primer design and efficiency calculation

Primers were designed for genes of interest based on the Affymetrix gene identifications. The specificity of each primer was verified by checking primer sequences against the National Center for Biotechnology Information (NCBI) Canine Genome using Primer-BLAST (Basic Local Alignment Search Tool) to identify possible false products. Primers were designed using Primer3 (Rozen and Skaletsky 2000) with a product size range of 80–150 bp. The Operon Oligo Analysis and Plotting Tool (Eurofins MWG Operon, Huntsville, AL) was used to check for primer dimers. Primers were ordered from Invitrogen (Carlsbad, CA). Primer efficiencies were calculated using a standard curve containing five 1:3 serial dilutions of template. A minimum efficiency of 1.9 was required for primers to be used in this study.

Quantitative RT-PCR

RNA extracted from tumor samples (five metastatic, five nonmetastatic) was converted to cDNA using the Qiagen

QuantiTect Reverse Transcription Kit. Quantitative RT-PCR was performed using the Applied Biosystems StepOne (Applied Biosystems, Carlsbad, CA), with SYBR® Green (Applied Biosystems) used as the fluorescent dye. The PCR cycle was followed with conditions as per the manufacturer's instructions (initial activation step of 10 min at 95°C, then 40 cycles of 95°C for 15 s and 60°C for 1 min), followed by a melt curve analysis to verify the number of products amplified. Total reaction volume was 20 µl (1 µl (50 ng) cDNA, 10 µl SYBR Green, 4 µl 1 µM primer stock, and 5 µl water). *β*-Actin was used as the endogenous control.

Quantitative RT-PCR data analysis

The Pfaffl method was used to determine the relative expression levels of the genes of interest. This method takes into account the variable efficiencies of the primer sets in qRT-PCR (Pfaffl 2001). Using this method, the expression ratio for each gene was calculated by comparing expression in metastatic tumors to expression in nonmetastatic tumors.

Results

Tumor samples were obtained from five dogs with metastatic and eight dogs with nonmetastatic STSs. Tumor type, grade, and mitotic index for each sample are given in Table 1. Results from the pooled microarray analysis are depicted in a cluster dendrogram (Fig. 1), which was generated by JMP/Genomics software. Over 200 genes that were differentially expressed between the metastatic and nonmetastatic groups were identified at a false discovery rate of 5%. The microarray data discussed in this article have been deposited in NCBI's Gene Expression Omnibus (Edgar et al. 2002) and are accessible through GEO Series accession number GSE24601 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE24601).

For validation of the microarray results, we chose five genes of interest that were more highly expressed in metastatic STSs than in the nonmetastatic group. All of these genes had at least a twofold greater expression in the metastatic group. Genes of interest were chosen based on known roles in cell division, transcription, or involvement in human cancers. These include *Sprouty2*, which prevents the degradation of epidermal growth factor receptor (EGFR) and has increased expression in malignant human fibroblasts; *Septin6*, which belongs to a family of GTP-binding proteins that are important in cytokinesis and has been implicated in human lymphoid neoplasia; G-protein signaling modulator 2 (*GPSM2*), which is involved in cell division via interaction with the nuclear mitotic apparatus protein; kinesin family member 23 (*KIF23*), a microtubule-dependent "motor" involved in the transport of organelles and chromosomes in cell division that has also been shown to interact with the inhibitor of apoptosis protein BIRC6 (BRUCE); and *SMAD homolog 9* (Mothers Against Decapentaplegic homolog 9), a member of the TGF-*β* superfamily of proteins and a regulator of transcription (Du and Yip 2010; Du et al. 2001; NCBI SEPT 6 2009, NCBI GPSM2 2009; Nislow et al. 1992; Pohl and Jentsch 2008; Seuntjens et al. 2009; Surka et al. 2002; Wong et al. 2001, 2002).

Primers were designed for these genes of interest, and primer sequence specificity was verified with Primer-BLAST to identify possible false products. All sequences were unique to the genes of interest with the exception of the *Sprouty2* primer, which also matched a glycine product of similar size.

Table 1 Tumor type, grade, and mitotic index for each of the 13 canine soft tissue sarcoma samples used in the microarray analysis

Sample	Tumor type	Tumor grade	Mitotic index	Metastatic or nonmetastatic
1	Fibrosarcoma	High	30	Metastatic
2	Fibrosarcoma	High	47	Metastatic
3	Fibrosarcoma	High	15	Metastatic
4	Fibrosarcoma	Low	2–5	Metastatic
5	Spindle cell sarcoma	High	42	Metastatic
6	Spindle cell sarcoma	Low	2	Nonmetastatic
7	Fibrosarcoma	Low	0	Nonmetastatic
8	Hemangiopericytoma	Low	3	Nonmetastatic
9	Hemangiopericytoma	Low	2	Nonmetastatic
10	Hemangiopericytoma	Low	1	Nonmetastatic
11	Hemangiopericytoma	Low	1	Nonmetastatic
12	Hemangiopericytoma	Low	6	Nonmetastatic
13	Hemangiopericytoma	Low	4	Nonmetastatic

Mitotic index is defined as the number of mitotic figures per 10 high-power microscope fields. Nonmetastatic tumor samples were from patients free of metastatic disease at least 1 year from the time of diagnosis

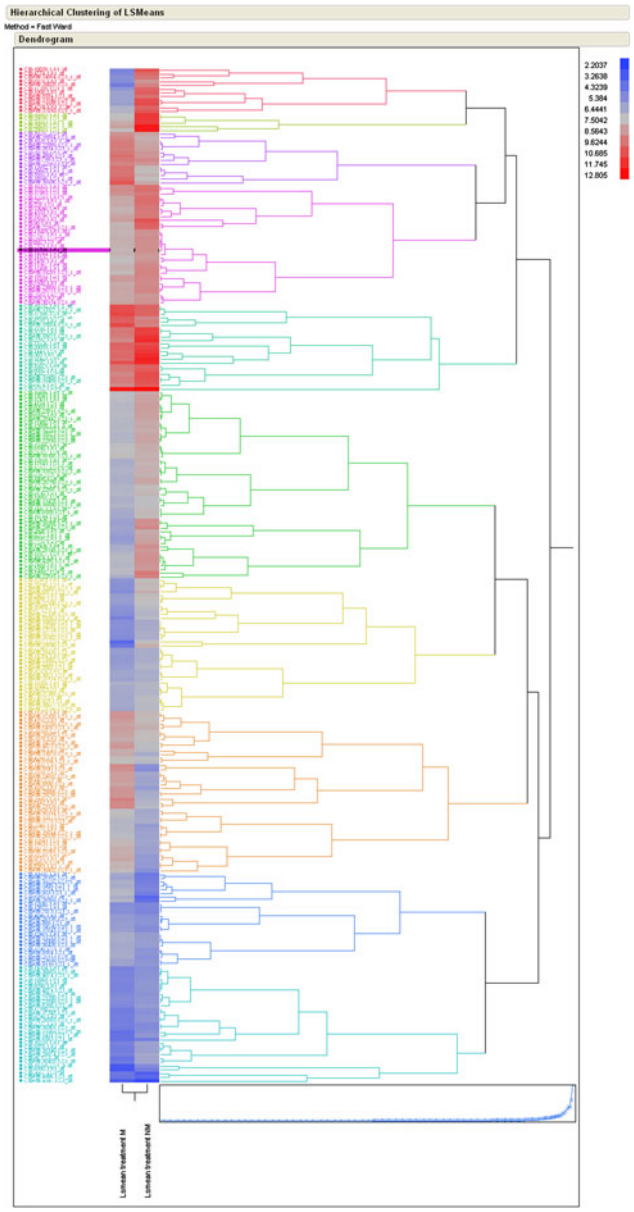

Fig. 1 Cluster dendrogram depicting results of the microarray analysis, generated by JMP/Genomics software. The cluster dendrogram was derived from the pooled microarray results of the metastatic and nonmetastatic tumor groups. Over 200 differentially expressed genes between the metastatic and nonmetastatic groups were identified at a false discovery rate of 5%. M represents the metastatic and NM represents the nonmetastatic tumor groups. Each *horizontal line* and *corresponding code* represent a single gene, with groupings (*color*) representing genes that are linked by common structure and/or function. The logarithmic scale to the *right* indicates relative gene expression, with darker shades of grey (*red*) indicating greater or lesser gene expression (Color figure online)

Validation with qRT-PCR was performed using the five metastatic tumor samples and five of the nonmetastatic tumor samples (samples 6, 8, 10, 11, 13). Duplicate PCR reactions were performed for each gene of interest. Duplicate reactions with a difference in CT value greater than 1 were excluded from analysis. Primer efficiencies used in the Pfaffl calculations are given in Table 2. Analysis with the Pfaffl method revealed increased relative expression of all five genes of interest in the metastatic STS group (Fig. 2). *Sprouty2* showed the greatest relative expression, with a 96-fold higher expression in metastatic than in nonmetastatic tumors. *SMAD homolog 4* was used as an internal negative control as it was not identified as a differentially expressed gene by the microarray. This gene did not show differential expression in the qRT-PCR analysis.

Table 2 Primer efficiencies

Primer (gene of interest)	Efficiency
Sprouty2	2.1838
Septin6	1.9206
GPSM2	1.915
KIF23	1.9771
SMAD9	1.9303
SMAD4	1.9545
β-actin	1.9571

Efficiencies were determined for the primers for each of the genes of interest, as well as for *β*-actin, the endogenous control in the qRT-PCR reactions

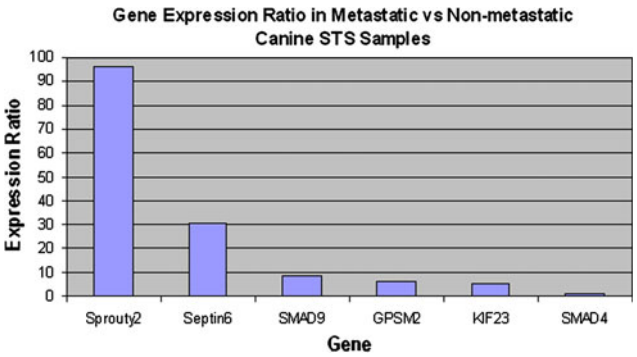

Fig. 2 Expression ratio of genes of interest in the metastatic tumor group relative to the nonmetastatic tumor group. Expression ratio was calculated using the Pfaffl method

Discussion

Given that the term "soft tissue sarcoma" includes a number of similarly behaving mesenchymal tumor types, it was unclear whether differences between tumor types would mask differences in gene expression between metastatic tumors and nonmetastatic tumors. Therefore, the five metastatic tumor samples and eight nonmetastatic tumor samples were selected initially for microarray analysis to determine the feasibility of this technique in this

setting. Real-time PCR results confirmed the results of the cDNA microarray. All five genes that were identified by the microarray to have greater expression in metastatic tumors were confirmed to have increased relative expression in the metastatic tumor group by qRT-PCR. Microarrays give a broad overview of tumor gene expression at the time of biopsy and are known to contain false positives, so it is helpful to confirm results with an independent method such as qRT-PCR. Quantitative RT-PCR results were analyzed using the Pfaffl method, which accounts for the varying efficiencies of the primer sets. This is important when evaluating genes that are expressed at relatively lower frequencies in a particular sample.

Five metastatic and five nonmetastatic canine STS tumor samples were used for qRT-PCR analysis. Three of the nonmetastatic tumors were not used because of limited availability of RNA. Our consistent results demonstrate that the cDNA microarray and qRT-PCR are feasible methods for evaluating gene expression in STSs, as has been demonstrated in various human studies (Baird et al. 2005; Landemaine et al. 2008; Nilbert et al. 2004; Ramaswamy et al. 2003).

Specificity of all of the primer sequences were verified against the canine genome using Primer-BLAST. The *Sprouty2* primer sequence also matched a glycine product of similar size. Therefore, while *Sprouty2* had considerably increased expression relative to the other genes of interest, its expression may have been artificially elevated due to the formation of a second product. In addition, the primer efficiency of *Sprouty2* was calculated as greater than 100% (2.1838), which should not be possible. Determination of relative expression using the Livak method (Schmittgen and Livak 2008), which assumes 100% primer efficiencies, reveals a relative expression of 48.5, which is more consistent with the expression levels of the other genes of interest.

Eight PCR reactions were excluded from Pfaffl analysis because the difference in duplicate CT values was greater than 1. These consisted of three *Sprouty2* and *GPSM2* reactions, as well as one *KIF23* and one *SMAD9* reaction. Possible explanations for the discrepancies in CT values could include pipetting errors, the formation of a second product, such as in the case of *Sprouty2*, or a contaminant in any of these reactions.

Initially, the primer for the *SMAD* gene was designed for the incorrect isoform (*SMAD4* instead of *SMAD9*). *SMAD4* was not identified as a differentially expressed gene on the microarray, and no difference in relative expression was found using RT-PCR. This further demonstrates the agreement between the microarray and RT-PCR results.

Our results demonstrate the feasibility of using the cDNA microarray and qRT-PCR to analyze gene expression in canine STSs, with the goal of eventually using these methods for evaluating patient tumor samples and predicting tumor metastasis. The presence of intertumor heterogeneity did not prevent identification of genes associated with the metastatic phenotype. In a clinical setting, qRT-PCR is the more cost-effective method for evaluating patient samples and therefore consistency of these results is critical. Evaluation of additional tumor samples is necessary to demonstrate the reproducibility of these results and to identify a unique gene signature that is predictive of metastasis. Future studies will also include pathway analysis to better delineate the roles of genes of interest in metastasis.

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