Validation of immature adipogenic status and identification of prognostic biomarkers in myxoid liposarcoma using tissue microarrays☆

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Summary Myxoid liposarcoma displays variably aggressive behavior and responds poorly to available systemic therapies. Expression profiling followed by tissue microarray validation linked to patient outcome is a powerful approach for validating biological mechanisms and identifying prognostic biomarkers. We applied these techniques to independent series of primary myxoid liposarcomas in an effort to assess markers of adipose differentiation in myxoid liposarcoma and to identify prognostic markers that can be efficiently assessed by immunohistochemistry. Candidate genes were selected based on analysis of expression profiles from 9 primary myxoid/round liposarcomas and 45 other soft tissue tumors, and by reference to publicly available data sets. Protein products were validated on an adipose neoplasm tissue microarray, including 32 myxoid liposarcomas linked to patient outcome. Results were scored visually and correlated with clinical outcome by Kaplan-Meier and Cox regression analyses. In the study, by examining expression patterns of several lipogenic regulatory gene products, an immature adipogenic status was verified in myxoid liposarcomas. We also found that expression levels of the ret proto-oncogene, insulin-like growth factor 1 receptor, and insulin-like growth factor 2 correlate with poor metastasis-free survival, supporting a role for ERK/MAPK and PI3K/AKT pathways in clinically aggressive myxoid liposarcomas.

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1. Introduction

Myxoid liposarcoma represents about 10% of adult soft tissue sarcomas and accounts for one third of liposarcomas [1]. Peak incidence is between 30 and 50 years of age. Proximal lower limbs and the retroperitoneum are the most common primary sites, and metastatic cases have a...
propensity to spread to unusual extrapulmonary locations [1,2]. Characteristic chromosomal translocations t(12;16) (FUS-DDIT3) or t(12;22) (EWS-DDIT3) are detectable in about 90% and 5% of myxoid liposarcoma cases, respectively [3,4], which can aid in diagnosis [5,6]. Primary myxoid liposarcoma with more than a 5% round cell component is associated with a poor clinical prognosis [7].

The fusion oncoprotein FUS-DDIT3 has been implicated in the initiation of myxoid liposarcoma, with its tumorigenic ability proven in different in vitro and in vivo model systems [8][9,10]. Mechanistic studies suggest that its oncogenic role is to block differentiation and bypass cell cycle controls [11,12]. Although in vitro studies have shown that FUS-DDIT3 blocks adipocyte differentiation by direct inhibition of CEBPβ, corresponding evidence from clinical tumor specimens is lacking. Expression profiling studies by our group and others have revealed a distinctive immature adipocytic signature in myxoid liposarcoma tumor specimens [13,14]. In the present study, we assess protein expression of key adipogenic regulatory molecules using a tissue microarray incorporating normal, benign, and malignant fatty tissues, aiming to validate the suggested differentiation blockade theory on clinical samples and to identify supporting prognostic biomarkers that can be detected by immunohistochemistry on standard pathology specimens. Currently, tumor grade (a function of differentiation, mitotic count, and necrosis) and stage (based on size and location, as well as grade) are the only frequently used criteria to determine patient prognosis and need for aggressive therapy. However, these factors do not take into account the specific underlying molecular biology of this disease. Using tissue microarrays, we compare expression of molecular biomarkers with patient outcome and identify 3 that are linked to poor prognosis in myxoid liposarcoma, unassociated with round cell morphology.

2. Materials and methods

2.1. Expression profiling of myxoid liposarcoma

Several candidate biomarkers of interest in myxoid liposarcoma were identified by analysis of microarray data from our group [13] (T. O. Nielsen and M. van de Rijn, unpublished observations) and from surveys of published microarray expression profiles of soft tissue tumors [14-17]. In this report, supporting microarray data are presented from a series including 9 cases of myxoid/round cell liposarcoma, 12 leiomyosarcomas, 9 desmoid-type fibromatoses, 12 synovial sarcomas, and 12 schwannomas. Mean log ratio intensity measurements were retrieved for the clones of interest from the Stanford Microarray Database (http://smd.stanford.edu/).

2.2. Tissue microarray construction and patient cohort

Thirty-two surgically excised primary myxoid liposarcomas, 9 well-differentiated liposarcomas, 10 lipomas, 6 pleomorphic liposarcomas, and 9 samples of normal fat were used to build the lipogenic tissue microarray. All these formalin-fixed, paraffin-embedded specimens were from surgically treated cases archived in Vancouver General Hospital from 1970 to 2005. Diagnoses for all these cases were reconfirmed, and representative areas in the source blocks were selected by an experienced sarcoma pathologist (T. O. N.) before tissue microarray construction. Patient clinical data were obtained for the myxoid liposarcoma cases, including information on disease recurrence, metastasis, and death, and are presented in Table 1. For each case, material obtained from nonradiated primary tumor tissue was specifically reviewed for the presence of a round cell component exceeding 5% [7] and was graded by the FNCLCC (Fédération Nationale des Centres de Lutte Contre le Cancer) system [18]. Median age at diagnosis was 44 years, and the included cases had no evidence of metastasis at the time of primary surgery. Follow-up data ranged from 4 to 209 months (median, 78 months), with the earliest healthy dropout at 25 months. One patient was lost to follow-up and was excluded from survival analyses. This study was approved by the Clinical Research Ethics Board of the University of British Columbia.

For tissue microarray construction, triplicate 600-μm cores were taken from different representative diagnostic areas from each paraffin-embedded tissue block and transferred to a recipient block using a Beecher Instruments (Sun Prairie, WI, USA) tissue arrayer, as described [19].

2.3. Immunohistochemical staining and scoring methods

Tissue microarray paraffin sections (4 μm) were stained with commercially available antibodies to the following proteins: PPARγ (sc-7273), DLK1 (sc-8624), RET (sc-1290), IGF1R (sc-713), 11βHSD2 (sc-19262), and CEBPα (sc-61) from Santa Cruz Biotechnology, Santa Cruz, CA; IGF2 (ab9574) and HPGD (ab37148) from Abcam, Cambridge, MA; and Ki67 (790-2910) from Ventana, Tucson, AZ. Slides were incubated with antibodies to PPARγ (at 1:50 dilution) and 11βHSD2 (1:50) overnight, after microwave heating–induced antigen retrieval and endogenous peroxidase blockage with 3% hydrogen peroxide in phosphate-buffered saline. Secondary antibody reactions were performed with peroxidase-labeled polymer conjugated to antimouse immunoglobulin G (Impress kit, MP-7402; Vector Laboratories, Burlingame, CA) or antigoat immunoglobulin G (Vector Laboratories). Detection was by EnVision+ (DAKO, Carpenteria, CA, USA) with diaminobenzidine chromogen, as per manufacturer’s protocol. For all
the remaining antibodies, immunostaining was performed using a Ventana Discovery automated immunostainer (Ventana Medical Systems). Heat-induced antigen retrieval was done with EDTA buffer (pH 8.0) for 24 minutes. Primary antibodies were incubated for 15 to 60 minutes at 37°C, at a concentration of 1:40 for RET, 1:15 for IGF1R, 1:50 for CEBPα, 1:20 for DLK1, 1:100 for IGF2, and 1:2000 for HPGD, and at the prediluted concentration for the Ki67 kit. Universal Discovery peroxidase-labeled antimouse/antirabbit secondary antibodies (Ventana Medical Systems) and antigoat secondary antibody (Vector Laboratories) (1:100) were used and developed for detection using the DAB Map Kit (Ventana Medical Systems). Hematoxylin counterstaining was carried out for all sections. Slide image data were digitally acquired using a Bacus Laboratories (Lombard, IL) Slide Scanner system, and the tissue microarray core images (5000) are available online at http://www.gepimage.ubc.ca/.

Staining results for all antibodies were scored by 2 sarcoma pathologists. For most antibodies, a 4-point scoring system was used, where 0 = staining above background in less than 10% of tumor cells, 1 = staining above background for 10% to 50% tumor cells, 2 = staining above background for 50% to 90% tumor cells, and 3 = staining above background for ≥ 90% of tumor cells. For Ki67 and HPGD, which stained smaller subsets of tumor cells, the scoring system used different cut points, where 0 = 0%, 1 = 1% to 10%, 2 = 10% to 20%, and 3 ≥ 20%. Positive PPARγ, CEBPα, and Ki67 stains were recorded only if immunostaining was seen within the nuclei of tumor cells, whereas DLK1, RET, IGF1R, IGF2, 11βHSD2, and HPGD were scored positive if cytoplasmic and/or membranous tumor cell staining was observed. Tissue cores were excluded if they failed to adhere to the glass slide or if they were considered unscoreable because of the presence of fewer than 50 tumor cells.

2.4. Statistical methods

The final score for each antibody was calculated by averaging the interpretable scores for the triplicate cores from each case. The tabulated results were then analyzed with the SPSS 15.0 statistical suite (SPSS Inc, Chicago, IL) and R 2.4.0 for Windows (http://www.r-project.org/). For survival analyses, the cutoff score was set as 2, meaning that, if the final averaged score for a case was less than 2 (corresponding to <50% positive cells for PPARγ, CEBPα, DLK1, RET, IGF1R, IGF2, 11βHSD2, and HPGD), the case was considered negative/low expresser for the biomarker; but if it was ≥ 2, the case was scored as positive. Metastasis-free survival curves were constructed by the Kaplan-Meier method and compared by the log-rank test for significant differences. Cox regression analysis was also performed for each prognostic factor to estimate hazard ratios for development of myxoid liposarcoma distant metastases. Multivariate analysis could not be performed because of the small number of patients with complete follow-up (31) and events (12) relative to the number of clinical and molecular biomarkers under assessment. Correlative analysis between biomarkers was done using the Fisher exact test (corrected using the Bonferroni-Holmes method [20]) and the Kendall test. Results were considered statistically significant if P value from a 2-tailed test was less than .05.
3. Results

3.1. Selection of candidate biomarkers

In gene profiling studies by our group [13] (T. O. Nielsen and M. van de Rijn, unpublished observations) and others [14-17], myxoid liposarcoma tumor specimens were found to display an immature adipogenic profile and markers of increased proliferation. From these data, 9 candidate genes were selected for the current study based on (a) differential expression level, (b) association with key adipogenic and proliferation pathways, and (c) the availability of antibodies suitable for immunohistochemistry on formalin-fixed, paraffin-embedded tissues. Among them, 5 are related to adipogenesis (PPARG, CEBPA, DLK1, HSD11B2, and HPGD) and 4 are proliferation-related genes (RET, IGF1R, IGF2, and MKI67). To visualize the relative expression level of selected genes, a heat map was generated by using complementary DNA data sets from 9 primary myxoid liposarcomas and 45 other soft tissue tumors (Fig. 1) from the Stanford Microarray Database.

PPARγ and CEBPα are well-recognized transcription factors central to the adipogenic differentiation program [21], in which PPARG is expressed at early stages of adipogenesis and CEBPA is expressed as adipocytes mature. Whereas PPARG was found to be highly expressed in myxoid liposarcoma, CEBPA is relatively low when compared with other soft tissue sarcomas (Fig. 1). DLK1 is also an important adipogenic regulator, thought to be secreted by preadipocytes, and has a role in inhibiting adipogenic differentiation [22]. Glucocorticoids represent a major physiologic stimulus for adipocyte differentiation [23], and the high levels of HSD11B2 expression are significant in this regard because the encoded enzyme inactivates cortisol [24]. HPGD has a similar pattern of differential expression, encoding an enzyme that catalyzes prostaglandin degradation; its activity may be related to PPARγ activation through regulation of endogenous PPARγ ligand production [25].

Genes influencing cell proliferation are also of obvious interest. We focused our analysis on biomarkers that are detectable by immunohistochemistry and may represent potential therapeutic targets (ie, tyrosine kinase receptors and their ligands). In this category, RET, IGF1R, and IGF2 were prominently expressed in the myxoid liposarcoma samples relative to other soft tissue tumors (Fig. 1). We also included the proliferation marker MKI67 in this category to assess overall proliferation status, although its expression is lower in myxoid liposarcoma than in synovial sarcoma and leiomyosarcoma (Fig. 1).

3.2. Tissue microarray immunohistochemical staining and scoring

Representative images are shown in Fig. 2, all primary images are available at http://www.gpecimage.ubc.ca/, and results are summarized in Table 2. All myxoid liposarcoma cases showed at least some PPARγ staining (100% sensitivity), whereas only 13 of 34 other specimens were positive (62% specificity for myxoid liposarcoma in comparison with the other adipose neoplasms and normal fat). As expected, most myxoid liposarcoma cases were negative for CEBPα; well-differentiated liposarcomas were the only entity where most cases stained for this marker. Somewhat unexpectedly, DLK1 did not stain the myxoid liposarcoma neoplastic cells themselves but rather stained...
the endothelium of the tumor microvasculature (Fig. 2). Both HPGD and 11\(\beta\)HSD2 were confirmed to be moderately to highly expressed in most myxoid liposarcoma specimens. Expression levels of Ki67 were much higher on myxoid liposarcomas than on normal fat samples, as was also true for RET, IGF1R, and IGF2. The highest levels of RET

**Table 2**  Tissue microarray immunohistochemical scoring results for myxoid liposarcoma, normal fat, well-differentiated liposarcoma, pleomorphic liposarcoma, and lipoma

<table>
<thead>
<tr>
<th>Antigen name</th>
<th>MLS (n = 32)</th>
<th>NF (n = 9)</th>
<th>PLS (n = 6)</th>
<th>WDLS (n = 9)</th>
<th>LP (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPAR(\gamma)</td>
<td>7</td>
<td>18</td>
<td>7</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>CEBP(\alpha)</td>
<td>23</td>
<td>7</td>
<td>2</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>DLK1</td>
<td>25</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>11(\beta)HSD2</td>
<td>15</td>
<td>6</td>
<td>7</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>HPGD</td>
<td>11</td>
<td>13</td>
<td>2</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>Ki67</td>
<td>13</td>
<td>15</td>
<td>15</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>RET</td>
<td>13</td>
<td>12</td>
<td>4</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>IGF1R</td>
<td>5</td>
<td>12</td>
<td>13</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>IGF2</td>
<td>18</td>
<td>9</td>
<td>3</td>
<td>2</td>
<td>9</td>
</tr>
</tbody>
</table>

**NOTE.** n = total scored cases of each entry disease; score value is the average of 3 cores as described in the “Materials and methods” section. Abbreviations: MLS, myxoid liposarcoma; NF, normal fat; WDLS, well-differentiated liposarcoma; PLS pleomorphic liposarcoma; LP, lipoma.
expression were observed in pleomorphic liposarcomas, which may suggest a particular association with this most aggressive of adipose neoplasms.

Myxoid liposarcoma patient demographics, treatment, and outcome data are presented in Table 1. Of the 31 with patients with complete follow-up data, 12 experienced distant relapse, which led to death in all these cases. Using distant metastasis-free survival as the clinical end point, we used Kaplan-Meier methodology with log-rank testing to assess the relationship of the assessed biomarkers with patient outcome over time.

RET, IGF1R, and IGF2 were significantly associated with poor metastasis-free survival (Fig. 3). The remaining biomarkers showed no significant prognostic value in the present study. To further estimate the survival probabilities and the cumulative hazard for the identified biomarkers, we also calculated hazard ratios using Cox regression. As shown in Table 3, in univariate analysis, RET, IGF1R, and IGF2 each correlated with significantly higher patient risk for developing metastasis after primary diagnosis. Among the clinicopathologic factors, tumor size of at least 10 cm and round cell component of at least 5% were significant adverse prognostic factors, with high FNCLCC grade of borderline

### Table 3

Univariate hazard ratios estimated with 95% confidence intervals by Cox regression analysis for metastasis-free survival in 31 myxoid liposarcoma patients

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Hazard ratio</th>
<th>95% CI</th>
</tr>
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<tbody>
<tr>
<td>IGF1R</td>
<td>7.44</td>
<td>1.59-34.8</td>
</tr>
<tr>
<td>IGF2</td>
<td>5.15</td>
<td>1.49-17.8</td>
</tr>
<tr>
<td>RET</td>
<td>3.65</td>
<td>1.06-12.6</td>
</tr>
<tr>
<td>Grade (low vs high)</td>
<td>3.82</td>
<td>0.99-14.8</td>
</tr>
<tr>
<td>Margins</td>
<td>1.60</td>
<td>0.50-5.02</td>
</tr>
<tr>
<td>Necrosis</td>
<td>2.53</td>
<td>0.77-8.38</td>
</tr>
<tr>
<td>Round cell</td>
<td>7.74</td>
<td>1.98-30.3</td>
</tr>
<tr>
<td>Tumor size ≥10 cm</td>
<td>9.83</td>
<td>1.25-77.1</td>
</tr>
</tbody>
</table>

Abbreviation: CI, confidence interval.

### Table 4

Correlation analysis among biomarkers and significant clinicopathologic prognostic factors

<table>
<thead>
<tr>
<th></th>
<th>RET</th>
<th>IGF2</th>
<th>IGF1R</th>
<th>Round cell</th>
<th>Tumor size</th>
</tr>
</thead>
<tbody>
<tr>
<td>RET</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P = .367</td>
<td>P = .755</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>τ = 0.895</td>
<td>P = 1</td>
</tr>
<tr>
<td>IGF2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P = .483</td>
<td>τ = 0.546</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>τ = 0.225</td>
<td>τ = 0.168</td>
</tr>
<tr>
<td>IGF1R</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P = .0825</td>
<td>P = .504</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>τ = 0.483</td>
<td>τ = .322</td>
</tr>
<tr>
<td>Round cell</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P = .903</td>
</tr>
<tr>
<td>Tumor size</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>τ = 0.174</td>
</tr>
</tbody>
</table>

NOTE. *P* values from Fisher exact test corrected for multiple comparisons by the Bonferroni-Holmes method and Kendall τ-b correlation coefficient are shown in the table.
4. Discussion

Several publications have suggested that myxoid liposarcoma is a neoplastic disease with defective adipogenesis [9,11] and that the FUS-DDIT3 fusion oncoprotein is the primary culprit in forestalling full adipogenic differentiation [11,12]. However, these findings were derived from in vitro models and have not been clearly assessed or validated in clinical patient samples. In this study, we investigated myxoid liposarcoma differentiation status by measuring several key adipogenic regulatory gene products on a patient sample cohort.

PPARγ and CEBPα are both well established as central transcription factors in adipogenesis [21,26]. PPARγ regulates lipid metabolism and insulin sensitivity, whereas CEBPα is normally expressed at the terminal differentiation stage, causing cell cycle exit and regulating glucose metabolism and insulin sensitivity [21,27]. In the current study, we found that PPARγ is overexpressed and CEBPα is underexpressed at both RNA and protein levels in myxoid liposarcoma. These findings are further supported by gene expression profiles that show numerous PPARγ target genes to be highly expressed, with insulin-responsive genes expressed at low levels relative to mature adipocytes [13,14].

DLK1 is a secreted factor that is expressed in preadipocytes and is known to suppress adipocyte differentiation in vitro [28]. High expression of DLK1 in myxoid liposarcoma (Fig. 1) led us to postulate that it may contribute to impaired differentiation in myxoid liposarcoma cells. However, immunohistochemical results showed that intense DLK1 staining was in fact localized to the microvascular endothelium rather than to the malignant cells. This finding suggests that the role for DLK1 in myxoid liposarcoma may be more related to regulation and patterning of angiogenesis.

Glucocorticoids are also known to modulate adipogenesis, and function to initiate differentiation and to inhibit proliferation in preadipocytes [29,30]. HSD11B2 and HSD11B1 encode 2 glucocorticoid-catalyzing enzymes with competing activities in determining intracellular glucocorticoid receptor activity [31]. High expression of HSD11B2 (but not HSD11B1, which is highly expressed in terminally differentiated adipocytes) further supports an incomplete differentiation status in myxoid liposarcoma. HPGD encodes another enzyme that catalyzes the degradation of prostaglandin, a major precursor of endogenous PPARγ ligands [25]. High expression of this gene’s product is also validated in the present study.

Using tissue microarray methodology, we were able to confirm protein expression of several proliferation-associated gene products on myxoid liposarcoma clinical specimens. We used a cut point of 50% of myxoid liposarcoma cells positive above background to define high-level biomarker expression in a manner applicable to visual assessment in clinical practice. We correlated high levels of protein expression with clinical outcome. RET, IGF1R, and IGF2 were found to be significantly associated with distant relapse in univariate analysis, as were presence of a round cell component of at least 5% and large tumor size. These latter 2 clinicopathologic parameters conferred the highest raw hazard ratios; however, apart from a weak correlation between IGF1R and round cell morphology, RET, IGF1R, or IGF2 expression did not correlate significantly with round cell status or tumor size, suggesting that these biomarkers may retain independent value. Validation and formal proof in a multivariable Cox model will require a larger, independent series.

RET is a receptor tyrosine kinase that plays a crucial role in neural crest development [32]. RET is usually activated by different neurotrophic factors and relays growth and differentiation signals from the cell environment. Activated RET can further activate proliferation and cell survival via the Ras-Raf-ERK/MAPK and the PI3K/AKT pathways. High expression of RET may thus contribute to tumor progression in myxoid liposarcomas and was also found in pleomorphic liposarcomas, the most aggressive of adipose neoplasms. IGF1R is another receptor tyrosine kinase we found to be highly expressed in myxoid liposarcoma; and IGF2, a major activating ligand for IGF1R, was also highly expressed at the RNA and protein levels. Akin to RET, IGF2-IGF1R signaling can activate the Ras-Raf-ERK/MAPK and the PI3K/AKT pathways [33], further supporting the involvement of these pathways in myxoid liposarcoma tumorigenesis and/or disease progression. These receptors and the pathways they activate are targeted by new generations of experimental therapeutics [34,35], which may have relevance for myxoid liposarcoma [34]. RET expression correlates with expression of the IGF pathway members, but these markers did not correlate significantly with the presence or absence of a round cell phenotype.

In conclusion, by validating the expression of several key adipogenic regulatory molecules, the present work supports the existence of an immature adipocytic phenotype in this myxoid liposarcoma. RET, IGF1R, and IGF2 are novel negative prognostic biomarkers that may imply a role in disease progression and may support targeted treatment strategies for aggressive cases.
References


