Expression Profiling of Liposarcoma Yields a Multigene Predictor of Patient Outcome and Identifies Genes That Contribute to Liposarcomagenesis

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Abstract

Liposarcomas are the most common type of soft tissue sarcoma but their genetics are poorly defined. To identify genes that contribute to liposarcomagenesis and serve as prognostic candidates, we undertook expression profiling of 140 primary liposarcoma samples, which were randomly split into training set (n = 95) and test set (n = 45). A multigene predictor for distant recurrence-free survival (DRFS) was developed by the supervised principal component method. Expression levels of the 588 genes in the predictor were used to calculate a risk score for each patient. In validation of the predictor in the test set, patients with low risk score had a 3-year DRFS of 83% versus 45% for high risk score patients (P = 0.001). The HR for high versus low score, adjusted for histologic subtype, was 4.42 (95% CI, 1.26–15.55; P = 0.021). The concordance probability for risk score was 0.732. In contrast, the concordance probability for histologic subtype, which had been considered the best predictor of outcome in liposarcoma, was 0.669. Genes related to adipogenesis, DNA replication, mitosis, and spindle assembly checkpoint control were all highly represented in the multigene predictor. Three genes from the predictor, TOP2A, PTK7, and CHEK1, were found to be overexpressed in liposarcoma samples of all five subtypes and in liposarcoma cell lines. RNAi-mediated knockdown of these genes in liposarcoma cell lines reduced proliferation and invasiveness and increased apoptosis. Taken together, our findings identify genes that seem to be involved in liposarcomagenesis and have promise as therapeutic targets, and support the use of this multigene predictor to improve risk stratification for individual patients with liposarcoma. Cancer Res; 71(7): 2697–705. ©2011 AACR.

Introduction

Liposarcoma is the most common soft tissue sarcoma, accounting for approximately 20% of cases (1). Liposarcoma is classified into 5 histologic subtypes that fall into 3 biological groups characterized by specific genetic alterations (1, 2): well-differentiated/dedifferentiated liposarcoma by amplification of the 12q13-15 chromosomal region (3), myxoid/round cell liposarcoma by chromosome 12 translocations (4), and pleomorphic liposarcoma by a complex karyotype (5, 6).

Surgery remains the primary treatment for localized liposarcoma, but approximately 40% of newly diagnosed patients will eventually die from advanced disease. Ifosfamide-based chemotherapy may improve survival for patients with round cell and pleomorphic liposarcoma (7), but it remains difficult to select those patients at highest risk of metastasis and most likely to benefit from such therapy. Liposarcoma subtype is the most important determinant of local recurrence, metastatic potential, and overall survival and has been used in a nomogram (2, 6, 8, 9); however, prediction of these outcomes is still imprecise for individual patients. In addition, there is a desperate need for new therapeutic approaches for patients with advanced liposarcoma, which often is resistant to conventional chemotherapy.

In this study we used microarray-based gene expression profiling to develop a multigene predictor that could predict an individual patient’s metastatic risk. Distant recurrence-free survival (DRFS) was the primary clinical endpoint. Selected upregulated genes predictive of liposarcoma metastasis were subjected to a functional analysis, enabling the identification of promising therapeutic targets.

Materials and Methods

Patient population and sample acquisition

The study cohort was patients treated for primary liposarcoma at Memorial Sloan-Kettering Cancer Center...
RNA extraction from primary liposarcoma tumors and U133A microarray analysis

After subtype was confirmed, primary tumors were macro-dissected to ensure subtype uniformity and to eliminate contamination. RNA was extracted from a cryomold (0.5 cm × 1 cm × 1 cm) for U133A microarray analysis. Specifically, each cryomold tumor was weighed and 1 mL of QIAzol lysis reagent added for every 100 mg of tumor. The cryomold tumor was homogenized by using the Mixer Mill MM 300 (Retsch, Inc.) and washed with ethanol. RNA was eluted by using the RNeasy Mini spin column from the RNeasy Lipid Tissue Mini Kit (Qiagen Inc.). cDNA was prepared by using oligo(dt)24-T7 (Genset Corp.), and cRNA was prepared by using biotinylated UTP and CTP. cRNA was hybridized to HG U133A oligonucleotide arrays (Affymetrix Inc.). The fluorescent signal was measured by laser confocal scanner (Agilent Technologies Inc.) and converted to signal intensity via the Affymetrix Microarray Suite V5 software. Gene expression data are available at the project Web site (10).

Statistical methods

U133A microarray results were processed by the robust multiarray average (RMA) method (11). The 140 primary liposarcoma samples were randomly split into a training set (n = 95) and a test set (n = 45) with stratification for length of follow-up and liposarcoma subtype. The supervised principal component method was used to generate the multigene predictor on a continuous scale from the training set data. The multigene predictor was then applied in the independent test set to calculate a genomic risk score (GRS) for each patient, using the R software and the superpc package (12). The statistical significance of GRS as predictor of DRFS was evaluated by using proportional hazard regression in univariate analysis and in multivariate analysis adjusting for histologic subtype. The predictiveness of GRS was evaluated by using the concordance probability computed by the phcpe package (13). The marginal effect of each gene in the multigene predictor was evaluated in the training set by using proportional hazards regression and was dichotomized at the median for generating the Kaplan–Meier curves.

Cell lines and cell culture

LPS141 and DLDS8817 dedifferentiated liposarcoma cell lines and RC5397 and ML2308 myxoid/round cell liposarcoma cell lines were previously established from human primary liposarcoma tumors. Liposarcoma cell lines were confirmed via SNP analysis to contain 12q amplification (LPS141, DLDS8817) and by PCR to contain a FUS/CHOP translocation (RC5397, ML2308). The adipocyte-derived stem cells (ASC) were previously established from human subcutaneous fat (14). Cell lines and ASCs were grown in a 50:50 mixture of Dulbecco’s modified Eagle’s medium (DMEM) high glucose and F12 media (DMEM HG/F12) with 10% FBS, 100 units/mL penicillin plus 100 μg/mL streptomycin (1× P/S) and maintained at 37°C in 5% CO₂.

Gene knockdown by shRNA lentiviruses

Human pLKO.1 lentiviral short hairpin RNA (shRNA) target gene sets (Thermo Scientific Open Biosystems) were individually tested for knockdown of TOP2A (in DDLS8817, ML2308), PTK7 (in LPS141), and CHEK1 (in DDLS8817, ML2308). Five distinct sequences per gene were assessed for knockdown, and the 2 that yielded greatest knockdown (listed in Supplementary Table 1) were used in the subsequent analyses. A scramble (SCR) sequence not known to target any human genes served as negative control. Viruses harboring the shRNA sequences were produced by transient cotransfection of 10 μg of shRNA lentiviral vector with 9 μg of the viral packaging gene pSPAX2 and 1 μg the viral envelope gene pMD2.G (Addgene) in a HEK 293T/17 cell line (ATCC) using Lipofectamine 2000 (Invitrogen). The infectious viral supernatants were collected in viral harvest medium (HG-DMEM + 10% FBS + 11 μg/mL bovine serum albumin + 1× P/S) at 48, 72, and 96 hours after transfection. Pooled viral supernatant was concentrated by using an Amicon Ultra-15 Centrifugal Filter Unit (Millipore).

For knockdown, cell lines of interest were infected with lentivirus by using polybrene (Sigma-Aldrich) to increase infection efficiency. Infected cells were selected with 1 μg/mL puromycin (Sigma-Aldrich). Expression of TOP2A, PTK7, CHEK1, and 18S rRNA was measured by quantitative real-time PCR (RT-PCR).

Quantitative real-time PCR

RNA was isolated from approximately 2.0 × 10⁷ cells of the LPS141, DDLS8817, RC5397, and ML2308 liposarcoma cell lines and ASCs by using the RNeasy Mini Kit (Qiagen). RNA (1.5 μg) was reverse transcribed by using TaqMan reverse transcription reagents (Applied Biosystems) in a Thermo Hydrod thermocycler (Thermo Hybaid). Quantitative real-time PCR (RT-PCR) was then performed with cDNA and TaqMan Gene Expression Assays (Applied Biosystems) by using the ABI Prism 7900HT Sequence Detection System. RT-PCR was analyzed with SDS version 2.1 software (Applied Biosystems). TaqMan Gene Expression Assays were used according to the manufacturer’s protocol to detect TOP2A (Hs03063307_m1), PTK7 (Hs00177173_m1), CHEK1 (Hs00176236_m1), and 18S rRNA (Hs99999901_s1). Relative gene expression was calculated by normalizing expression of TOP2A, PTK7, and CHEK1 to the expression of endogenous control, 18S rRNA.

Immunoblotting

Liposarcoma cell lines and ASCs were collected and lysed. Cell lysates containing 30 μg of protein were resolved by SDS-PAGE, transferred onto Immun-Blot PVDF Membrane...
(Bio-Rad Laboratories) and probed with antibody for TOP2A (sc-56803; Santa Cruz Biotechnology), CHEK1 (sc-81227; Santa Cruz Biotechnology), and α-tubulin (CP06; Calbiochem). Detection was performed with the Amersham ECL Plus Western blotting detection system per the manufacturer’s instructions (GE Healthcare).

**Proliferation assay**

Proliferation was assessed by measurement of DNA content. After lentivirus infection, approximately 10^5 cells were plated in 96-well plates, and cells were fixed 2 and 6 days after infection. DNA was quantified by using CyQuant Cell Proliferation kit (Invitrogen), and plates were read by using the Spectramax M2 fluorescence microplate reader (Molecular Devices) at 480/520 nm excitation/emission. Fold proliferation was calculated by dividing DNA content at day 6 by DNA content at day 2.

**Apoptosis assay**

The percentage of cells undergoing apoptosis after TOP2A, PTK7, and CHEK1 knockdown was measured by the Guava Nexin Kit (Guava Technologies) and compared with SCR. Briefly, lentivirus-infected cells were plated in triplicate in 6-well plates. On days 2 and 6, floating and adherent cells were collected and washed once with cold PBS and once with 1× Annexin V binding buffer. Cells were then stained with Annexin V and allowed to incubate at room temperature for 20 minutes. Cells positive for Annexin V were counted by the Guava Personal Cytometer and analyzed by Guava software to determine the percentage of cells undergoing early apoptosis and late apoptosis.

**Invasion assay**

Cell invasion into Matrigel membranes was measured in triplicate. Twenty-four–well BD BioCoat Matrigel Invasion membranes were rehydrated for 2 hours in a humidified tissue culture incubator (37°C, 5% CO₂), and 5.0 x 10^5 cells/mL of each liposarcoma cell line and ASCs were prepared in media without chemoattractant (DMEM HG/F12 + 1% P/S). Chemotactrant (DMEM HG/F12 + 10% FBS + 1% P/S) was added to the bottom of each well, and the cell suspension was added to the Matrigel Invasion Chambers and control inserts without Matrigel, then the units were incubated for 24 hours. The percentage of cells undergoing apoptosis after TOP2A, PTK7, and CHEK1 knockdown was measured by the Guava Nexin Kit (Guava Technologies) and compared with SCR.

The multigene predictor identified 586 genes whose expression correlated with DRFS (Supplementary Data). Increased DRFS was associated with 202 probe sets corresponding to 159 genes, whereas decreased DRFS was associated with 527 probe sets corresponding to 429 genes. On the basis of expression of these 586 genes, a GBS was calculated for each patient’s tumor. In the training set, patients with low GBS (below the median) had a 3-year DRFS of 88% compared with 43% for high GRS patients (on univariate analysis, P < 0.001; Supplementary Data).

**Results**

**Patient and tumor characteristics**

In total, 140 patients with primary liposarcoma tumors were used to generate and evaluate the multigene predictor. Patients’ characteristics are shown in Table 1. The most common histologic subtype was well differentiated (52 patients; 37.1%), followed by dedifferentiated (39 patients; 27.9%).

**Survival analysis**

Median follow-up was 44.1 months (range, 1.45–140.8) for survivors without distant recurrence. At last follow-up, 91 patients (65.0%) were alive and without evidence of distant recurrence, 30 patients had distant recurrence (21.4%), and 19 patients were dead without having distant recurrence (13.6%). The multigene predictor identified 588 genes whose expression correlated with DRFS (Supplementary Data). Increased DRFS was associated with 202 probe sets corresponding to 159 genes, whereas decreased DRFS was associated with 527 probe sets corresponding to 429 genes. On the basis of expression of these 586 genes, a GBS was calculated for each patient’s tumor. In the training set, patients with low GBS (below the median) had a 3-year DRFS of 88% compared with 43% for high GRS patients (on univariate analysis, P < 0.001; Supplementary Data).

<table>
<thead>
<tr>
<th>Patient Characteristic</th>
<th>n</th>
<th>% of total</th>
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<tr>
<td>Age, median (range), y</td>
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<td>42.1</td>
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<tr>
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<tr>
<td>Female</td>
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<tr>
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<td>57.9</td>
</tr>
<tr>
<td>Histologic variant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well-differentiated</td>
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</tr>
<tr>
<td>Dedifferentiated</td>
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<td>27.9</td>
</tr>
<tr>
<td>Myxoid</td>
<td>17</td>
<td>12.1</td>
</tr>
<tr>
<td>Round cell</td>
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<td>8.6</td>
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<tr>
<td>Pleomorphic</td>
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<td>14.3</td>
</tr>
<tr>
<td>Primary site</td>
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<td></td>
</tr>
<tr>
<td>Lower extremity</td>
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</tr>
<tr>
<td>Upper extremity</td>
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</tr>
<tr>
<td>Trunk</td>
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<td>2.9</td>
</tr>
<tr>
<td>Retroperitoneum</td>
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<td>Thorax</td>
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<td>3.5</td>
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<tr>
<td>Tumor depth</td>
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<tr>
<td>Superficial</td>
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<tr>
<td>Deep</td>
<td>136</td>
<td>97.1</td>
</tr>
<tr>
<td>Tumor burden, median</td>
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<td>62.9</td>
</tr>
<tr>
<td>(range), cm</td>
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<td>Margins</td>
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<tr>
<td>Positive micromargins</td>
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<td>32.8</td>
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<tr>
<td>Positive gross margins</td>
<td>6</td>
<td>4.3</td>
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</table>
Fig. 1A). After subtype was adjusted for, the HR for high versus low GRS was 4.33 (95% CI, 1.70–11.03; P = 0.002). In the independent test set, low GRS patients had a 3-year DRFS of 83% versus 45% for high GRS patients (P = 0.001; Supplementary Fig. 1B). The HR for high versus low GRS was 4.42 (95% CI, 1.26–15.55; P = 0.021) after subtype was adjusted for.

To determine whether a smaller subset of genes could accurately predict DRFS, the analysis was repeated with the number of probe sets in the predictor restricted to less than 100. This second analysis identified 12 probe sets corresponding to 11 genes that accurately predicted DRFS (Table 2). In the training set, patients with a low GRS from the 11-gene predictor had a 3-year DRFS of 93% versus 45% for high GRS patients (P = 0.004; Fig. 1B). The HR for high versus low GRS was 4.42 (95% CI, 1.70–11.03; P = 0.001) after subtype was adjusted for.

Selection of genes for further functional validation

We undertook further functional analysis of a few genes for which overexpression was associated with shorter DRFS and which are involved in potentially biologically relevant pathways so as to yield promising therapeutic targets. TOP2A and PTK7 were chosen because they were identified in both the 588-gene predictor and the 11-gene predictor and because of strong univariate associations with DRFS. CHEKI was chosen because it had one of the greatest marginal effects (univariate HR = 4.16, 95% CI, 1.85–8.24) and was found in 5 of the top 10 canonical pathways (Supplementary Table 2), as these genes may have a greater impact in metastasis of liposarcoma.

Table 2. Genes from the 11-gene predictor

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Function</th>
<th>Univariate HRa</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADH1B</td>
<td>Alcohol dehydrogenase 1B</td>
<td>Ethanol oxidation</td>
<td>0.77</td>
</tr>
<tr>
<td>ADIPOQ</td>
<td>Adiponectin</td>
<td>Regulation of glucose and lipid metabolism</td>
<td>0.78</td>
</tr>
<tr>
<td>ANK2</td>
<td>Ankyrin 2</td>
<td>Cell motility and proliferation</td>
<td>0.53</td>
</tr>
<tr>
<td>GPD1</td>
<td>Glycerol-3-phosphate dehydrogenase 1</td>
<td>Glycolytic pathway enzyme</td>
<td>0.61</td>
</tr>
<tr>
<td>NTRK2</td>
<td>Neurotrophic tyrosine kinase receptor type 2</td>
<td>Signal transduction and cellular differentiation</td>
<td>0.74</td>
</tr>
<tr>
<td>PLIN</td>
<td>Perilipin</td>
<td>Inhibition of lipolysis</td>
<td>0.72</td>
</tr>
<tr>
<td>RBP4</td>
<td>Retinol binding protein 4</td>
<td>Carrier for retinol in the blood</td>
<td>0.77</td>
</tr>
<tr>
<td>MMP14</td>
<td>Matrix metalloproteinase 14</td>
<td>Breakdown of extracellular matrix</td>
<td>2.84</td>
</tr>
<tr>
<td>PTK7</td>
<td>Protein tyrosine kinase 7</td>
<td>Extracellular signal transduction and cell adhesion</td>
<td>4.16</td>
</tr>
<tr>
<td>RAB23</td>
<td>RAB23, member RAS oncogene family</td>
<td>GTPase-mediated signal transduction and intracellular protein transport</td>
<td>1.85</td>
</tr>
<tr>
<td>TOP2A</td>
<td>Topoisomerase II alpha</td>
<td>Alteration of DNA topologic state during transcription</td>
<td>1.70, 1.85b</td>
</tr>
</tbody>
</table>

aHR > 1 implies that increased expression is associated with shorter DRFS.
bTOP2A has 2 HRs that correspond to 2 different probe sets.

Ingenuity pathway analysis

Genes from the 588-gene predictor are involved in a diverse range of functions important in adipogenesis (ADIPOQ, CEBPA, FABP4, LEP, LIPE, LPL, PLIN) and cancer development including DNA replication (CCNA2, CHEKI, TOP2A), spindle assembly checkpoint control (BUB1B, CDC20, MAD2L1, PLK1), angiogenesis (VEGF), apoptosis (BAX, BIRC3, SOX4), tumor growth (KRAS, PLAU), and tumor invasion (CDKN2A, CXCL12, MMP14), among others. To identify biologically relevant pathways, we performed an Ingenuity Pathway Analysis of the 588 genes. These genes fell into multiple canonical pathways; Table 3 shows the 10 canonical pathways with the highest estimated probability of involvement, and Supplementary Table 2 shows the genes from the 588-gene predictor in those pathways. Particularly interesting are the 15 genes that were found in more than 1 of the top 10 canonical pathways (Supplementary Table 2), as these genes may have a greater impact in metastasis of liposarcoma.
3-year DRFS was significantly worse for patients with high-expressing tumors than for patients with low-expressing tumors: 45% versus 91% for \textit{TOP2A}, 49% versus 87% for \textit{PTK7}, and 49% versus 87% for \textit{CHEK1} in univariate analysis (all $P < 0.001$). After histologic subtype was adjusted for, expression of \textit{TOP2A} (HR $= 1.55$; 95% CI, 1.04–2.29), \textit{PTK7} (HR $= 3.71$; 95% CI, 1.84–7.50), and \textit{CHEK1} (HR $= 4.39$; 95% CI, 1.37–14.06) each remained an independent predictor of decreased DRFS.

Expression of \textit{TOP2A}, \textit{PTK7}, and \textit{CHEK1}

In a comparison of gene expression in primary liposarcoma tumor samples versus normal fat using U133A Affymetrix gene arrays, \textit{TOP2A}, \textit{PTK7}, and \textit{CHEK1} all had elevated expression in all the liposarcoma subtypes (Supplementary Table 3). In addition, PCR was used to evaluate expression of these genes in our liposarcoma cell lines and in ASCs. We found increased expression in all 4 liposarcoma cell lines for \textit{TOP2A} (32- to 77-fold; all $P < 0.05$; Supplementary Fig. 2A) and, to a lesser extent, for \textit{CHEK1} (2.4- to 3.0-fold; all $P < 0.05$; Supplementary Fig. 2C). \textit{PTK7} expression was increased in LPS141, RC3397, and ML2308 cells (2.4- to 3.3-fold; all $P < 0.05$), but not in DDLS8817 cells (Supplementary Fig. 2B). Immunoblotting confirmed upregulation of \textit{TOP2A} and \textit{CHEK1} protein expression (Supplementary Fig. 2D).

shRNA knockdown of \textit{TOP2A}, \textit{PTK7}, and \textit{CHEK1}

We undertook knockdown studies of \textit{TOP2A}, \textit{PTK7}, and \textit{CHEK1} in liposarcoma cell lines in which the genes were highly overexpressed, with ASCs as control. Knockdown with the \textit{TOP2A}$\#7$ shRNA, compared with control SCR shRNA, decreased \textit{TOP2A} mRNA levels in ASCs, DDLS8817, and ML2308 by 93% to 98% (all $P < 0.01$ for \textit{TOP2A}$\#7$ vs. SCR; Supplementary Fig. 3A). Similarly, the \textit{PTK7}$\#7$ shRNA decreased \textit{PTK7} expression in ASCs (85%) and LPS141 (94%), and the \textit{CHEK1}$\#4$ shRNA decreased \textit{CHEK1} expression by 95% to 98% in ASCs, DDLS8817, and ML2308 (all $P < 0.001$; Supplementary Fig. 3B and C). A second shRNA construct for \textit{TOP2A} and \textit{PTK7} yielded similar levels of \textit{TOP2A} and \textit{PTK7} knockdown. However, the second \textit{CHEK1} construct yielded significantly weaker \textit{CHEK1} knockdown (Supplementary Fig. 3C).

Decreased expression of \textit{TOP2A} and \textit{CHEK1} after shRNA knockdown in the liposarcoma cell lines was confirmed by immunoblotting (Supplementary Fig. 3D).

### Table 3. Top 10 canonical pathways from Ingenuity pathway analysis

<table>
<thead>
<tr>
<th>Canonical pathway</th>
<th>$P$</th>
<th>Genes in pathway$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitotic roles of Polo-like kinase</td>
<td>$2.9 \times 10^{-2}$</td>
<td>12</td>
</tr>
<tr>
<td>Pyruvate metabolism</td>
<td>$3.4 \times 10^{-4}$</td>
<td>9</td>
</tr>
<tr>
<td>G2/M DNA damage checkpoint regulation</td>
<td>$3.4 \times 10^{-3}$</td>
<td>7</td>
</tr>
<tr>
<td>p53 signaling</td>
<td>$9.5 \times 10^{-4}$</td>
<td>10</td>
</tr>
<tr>
<td>Aryl hydrocarbon receptor signaling</td>
<td>$1.1 \times 10^{-3}$</td>
<td>13</td>
</tr>
<tr>
<td>Factors promoting cardiogenesis in vertebrates</td>
<td>$7.1 \times 10^{-3}$</td>
<td>8</td>
</tr>
<tr>
<td>Role of BRCA1 in DNA damage response</td>
<td>$7.9 \times 10^{-3}$</td>
<td>6</td>
</tr>
<tr>
<td>Glycolysis–gluconeogenesis</td>
<td>$8.2 \times 10^{-3}$</td>
<td>8</td>
</tr>
<tr>
<td>ATM signaling</td>
<td>$8.7 \times 10^{-3}$</td>
<td>6</td>
</tr>
<tr>
<td>Mechanism of viral exit from host cells</td>
<td>$1.0 \times 10^{-2}$</td>
<td>5</td>
</tr>
</tbody>
</table>

$^a$Number of genes from the 588-gene predictor.
Proliferation after shRNA knockdown

shRNA knockdown of TOP2A, compared with SCR, decreased proliferation of DDLS8817 (57.8% ± 10.7%, \( P < 0.01 \)) and ML2308 (38.1% ± 2.7%, \( P < 0.001 \)) by day 6 after infection (Fig. 3A). TOP2A knockdown did not, however, affect proliferation of ASCs. Similarly, shRNA knockdown of PTK7 decreased proliferation of LPS141 (36.8% ± 5.4%, \( P < 0.01 \)) but not ASCs. Similar decreases in proliferation were observed with TOP2A and PTK7 knockdown, using a second shRNA construct (Supplementary Fig. 4A and B). shRNA knockdown of CHEK1 with the CHEK1#4 shRNA compared with SCR decreased proliferation of DDLS8817 (71.8% ± 15.3%, \( P < 0.001 \)) and ML2308 (34.6% ± 14.3%, \( P < 0.01 \)) but not ASCs (Fig. 3A). Knockdown with the CHEK1#1 construct had a similar effect on proliferation of ML2308, but much less effect on proliferation of DDLS8817 than did CHEK1#4.

Late apoptosis after shRNA knockdown

Late apoptosis (day 6) was increased with the TOP2A#7 construct compared with SCR in DDLS8817 (43.4% vs. 13.6%, \( P < 0.001 \)) and in ML2308 (34.6% vs. 13.2%, \( P < 0.001 \); Fig. 3B). Similarly, treatment with the PTK7#7 construct compared with SCR increased late apoptosis of LPS141 (29.3% vs. 9.9%, \( P < 0.001 \)), and treatment with the CHEK1#4 construct compared with SCR increased late apoptosis of DDLS8817 (34.2% vs. 10.9%) and ML2308 (24.7% vs. 14.1%; \( P < 0.001 \); Fig. 3B).

Invasiveness after shRNA knockdown

To study the effects of shRNA knockdown of TOP2A, PTK7, and CHEK1 on invasiveness, we first tested whether our liposarcoma cell lines are invasive. Both our dedifferentiated liposarcoma lines (LPS141, DDLS8817) and round cell liposarcoma lines (RC5397, ML2308) were highly invasive; they ranged from 6.8- to 8.3-fold more invasive than the negative control (ASCs; \( P < 0.001 \); Supplementary Fig. 6).

For evaluating the effects of knockdown on invasiveness, we assessed invasiveness at 2 days after infection because neither apoptosis nor proliferation was significantly affected by knockdown by this time point. shRNA knockdown of TOP2A and CHEK1 significantly decreased the invasiveness of both DDLS8817 and ML2308 (all \( P < 0.01 \); Fig. 3C). Similarly, shRNA knockdown of PTK7 decreased the invasiveness of LPS141 (\( P < 0.001 \)). A similar decrease in invasion was observed with TOP2A, PTK7, and CHEK1 knockdown by using a second shRNA construct (Supplementary Fig. 7).

Discussion

Liposarcomas have highly variable metastatic potential. In an effort to improve the ability to predict risk of metastasis for individual patients, we generated and validated a multigene predictor, which uses expression of 588 genes to identify
patients at low versus high risk of metastasis. In external validation, the multigene predictor accurately predicted worse 3-year DRFS for those patients with high GRS tumors than for those with low GRS tumors with a HR of 4.4 (95% CI, 1.3–15.6).

We also developed and validated a smaller predictor consisting of 11 genes. Despite including only one-fiftieth as many genes as multigene predictor, the smaller gene predictor accurately segregated patients into high- and low-risk subgroups. Both predictors had higher concordance probability than the liposarcoma subtype, though the concordance probability was highest when the GRS was combined with subtype.

A nomogram developed specifically for liposarcoma includes liposarcoma subtype (8, 15). Adding GRS from the multigene predictor to the liposarcoma nomogram could plausibly allow more accurate determination of an individual patient’s risk. We note, however, that the liposarcoma nomogram used an endpoint of disease-specific survival, whereas we used DRFS.

Gene expression profiling can be useful in identifying potential therapeutic targets (2, 16, 17). On the basis of the association of TOP2A, PTK7, and CHEK1 with reduced DRFS, we conducted functional analyses of these genes. Expression of all 3 genes was elevated in primary liposarcomas and in most or all of our 4 liposarcoma cell lines. shRNA knockdown of these 3 genes confirmed their importance in liposarcoma cell lines. For all 3 genes, knockdown decreased cell proliferation and invasiveness and increased apoptosis.

Figure 3. Effects of TOP2A, PTK7, and CHEK1 knockdown in ASCs and liposarcoma cell lines. A, cell proliferation following shRNA knockdown relative to SCR at day 6 after infection. B, apoptosis at day 6 after infection, assessed by the percentage of cells staining for Annexin V. C, percentage invasion of liposarcoma cell lines at day 2 after lentivirus infection.
Knockdown of these genes did not affect proliferation or apoptosis in ASCs, so the effects were specific to liposarcoma cell lines.

TOP2A, a topoisomerase, regulates the topologic state of DNA during processes such as transcription. TOP2A expression is increased in multiple cancers (18–22), and overexpression is associated with aggressive biological behavior, advanced stage, and poor patient survival in epithelial cancer types (20–24). TOP2A is a target of anthracycline-based chemotherapy agents, which have been used to treat metastatic liposarcoma, albeit with limited effectiveness (7, 25, 26). However, anthracyclines and most other current TOP2A poisons have relatively poor potency for TOP2A (27). The present results suggest that rational screening for more potent and specific TOP2A-targeted agents may lead to more effective therapy for patients with liposarcoma.

PTK7 belongs to the receptor tyrosine kinase family, although it lacks kinase activity (28). PTK7 is involved in neural crest migration (29) and angiogenesis, apparently by contributing to the migration and invasion of endothelial cells (30). PTK7 is also believed to play other important roles during embryologic development (31). PTK7 is increased in cancers of the colon (32), stomach (33), and blood (34) and has been associated with metastasis in colorectal cancer (35). These results, when combined with our findings of the importance of PTK7 in liposarcoma cell lines and its association with liposarcoma metastasis, suggest that targeting of PTK7 through neutralizing antibodies or siRNA may be valuable as a therapeutic to inhibit both angiogenesis and liposarcomagenesis.

CHEK1 is involved in the control of the G2/M checkpoint, DNA repair, and resistance to radiation and chemotherapy (36). Aberrant CHEK1 expression has been shown in mesothelioma (36), pancreatic cancer (37), colorectal cancer (38, 39), and breast cancer (40). CHEK1 overexpression was associated with advanced tumor stage and worse prognosis in colorectal cancer. In addition, treatment of colon cancer xenografts with DNA-damaging agents upregulated CHEK1 expression and inhibited apoptosis (41). In a conditional Chek1 mutant mouse, proliferating mammary cells lacking CHEK1 undergo apoptosis (42). Taken together with our findings that CHEK1 knockdown induces apoptosis in liposarcoma cells, these results suggest an antiapoptotic role for CHEK1 in liposarcomagenesis and that liposarcomas that overexpress CHEK1 may be less responsive to DNA-damaging agents. Thus, combining DNA damaging agents with CHEK1 inhibitors may be a therapeutically promising approach in liposarcoma with upregulated CHEK1.

We have developed a multigene predictor that is more prognostic for DRFS than the liposarcoma subtype alone, which had been considered the best predictor of metastatic risk. We also developed a smaller gene predictor consisting of 11 genes that performed similarly to the larger multigene predictor. Three genes from the multigene predictor (TOP2A, PTK7, and CHEK1) were functionally validated via shRNA knockdown, which decreased proliferation and invasion and increased apoptosis in our liposarcoma cell lines but not in adipose-derived stem cells. These results suggest that TOP2A, PTK7, and CHEK1 are important drivers of liposarcomagenesis. These and other genes identified from this multigene predictor may serve as candidates for targeted therapeutics.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

We thank the members of the MSKCC Genomics Core Laboratory and Alisa Pinkhasik for clinical data support.

**Grant Support**

This work was supported by the National Cancer Institute at the National Institutes of Health through a Soft Tissue Sarcoma Program Project grant: P01 CA041779 to S. Singer and L.-X. Qin. This work was also partially supported by the Kristen Ann Carr Fund and a generous donation from Richard Hanlon.

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Received October 4, 2010; revised December 9, 2010; accepted January 30, 2011; published OnlineFirst February 18, 2011.

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_Cancer Res_ 2011;71:2697-2705. Published OnlineFirst February 18, 2011.

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