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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 second most common type of soft tissue sarcoma but its 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 multi-gene predictor for DRFS was developed using 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% vs. 45% for high risk score patients (P=0.001). The hazard ratio for high vs. low score, adjusted for histologic subtype, was 4.42 (95% confidence interval 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 multi-gene 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 appear to be involved liposarcomagenesis and have promise as therapeutic targets, and support the use of this multi-gene predictor to improve risk stratification for individual patients with liposarcoma.

Keywords

liposarcoma; distant recurrence–free survival; apoptosis; cell proliferation; cell invasion

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Introduction

Liposarcoma is the most common soft tissue sarcoma, accounting for about 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 multi-gene 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.

Methods

Patient population and sample acquisition

The study cohort was patients treated for primary liposarcoma at Memorial Sloan-Kettering Cancer Center (MSKCC) between August 1993 and June 2008. The study was approved by MSKCC's institutional review board. Data for each patient on liposarcoma subtype, treatment, time to distant metastasis, and survival were collected in a prospectively maintained database. Liposarcoma subtypes were assigned based upon morphologic features, and each assignment was reviewed by an expert sarcoma pathologist (Christina Antonescu).

RNA extraction from primary liposarcoma tumors and U133A microarray analysis

After subtype was confirmed, primary tumors were macrodissected 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 using the Mixer Mill MM 300 (Retsch, Inc., Newtown, PA) and washed with ethanol. RNA was eluted using the RNeasy Mini spin column from the RNeasy Lipid Tissue Mini Kit (QIAGEN Inc., Valencia, CA). cDNA was prepared using oligo(dT)24-T7 (Genset Corp., San Diego, CA), and cRNA was prepared using biotinylated UTP and CTP. cRNA was hybridized to HG U133A oligonucleotide arrays (Affymetrix Inc., Santa Clara, CA). The fluorescent signal was measured by laser confocal scanner (Agilent Technologies Inc., Santa Clara, CA) and converted to signal intensity via the Affymetrix Microarray Suite V5 software. Gene expression data are available at the project web site (10).

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Statistical methods

U133A microarray results were processed using the 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 multi-gene predictor on a continuous scale from the training set data. The multi-gene 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 using proportional hazard regression in univariate analysis and in multivariate analysis adjusting for histologic subtype. The predictiveness of GRS was evaluated using the concordance probability computed using the phcpe package (13). The marginal effect of each gene in the multi-gene predictor was evaluated in the training set using proportional hazards regression and was dichotomized at the median for generating the Kaplan-Meier curves.

Cell lines and cell culture

LPS141 and DDLS8817 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, DDLS8817) and by PCR to contain a FUS/CHOP translocation (RC5397, ML2308). The adipocyte-derived stem cells (ASCs) were previously established from human subcutaneous fat (14). Cell lines and ASCs were grown in a 50/50 mixture of DMEM high glucose and F12 media (DMEM HG/F12) with 10% fetal bovine serum (FBS), 100 units/mL penicillin plus 100 μg/mL streptomycin (1X P/S) and maintained at 37°C in 5% CO_2.

Gene knockdown by shRNA lentiviruses

Human pLKO.1 lentiviral short-hairpin RNA (shRNA) target gene sets (Thermo Scientific Open Biosystems, Huntsville, AL) 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 two 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, Cambridge, MA) in a HEK 293T/17 cell line (ATCC, Manassas, VA) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The infectious viral supernatants were collected in viral harvest medium (HG-DMEM + 10% FBS + 11 μg/mL BSA + 1X P/S) at 48, 72, and96 hours after transfection. Pooled viral supernatant was concentrated using an Amicon Ultra-15 Centrifugal Filter Unit (Millipore, Billerica, MA).

For knockdown, cell lines of interest were infected with lentivirus using polybrene (Sigma-Aldrich, St. Louis, MO) 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 2.0×10^7 cells of the LPS141, DDLS8817, RC5397, and ML2308 liposarcoma cell lines and ASCs using the RNeasy Mini Kit (Qiagen). RNA (1.5 μg) was reverse transcribed using TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA) in a Thermo Hybaid thermocycler (Thermo Hybaid, Waltham, MA). Quantitative real-time reverse transcription PCR (RT-PCR) was then performed with

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cDNA and TaqMan Gene Expression Assays (Applied Biosystems) 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 Immuno-Blot polyvinylidene fluoride membranes (Bio-Rad Laboratories, Hercules, CA) and probed with antibody for TOP2A (sc-56803, Santa Cruz Biotechnology, Santa Cruz, CA), CHEK1 (sc-81227, Santa Cruz Biotechnology), and α-tubulin (CP06, Calbiochem, San Diego, CA). Detection was performed with the Amersham ECL Plus western blotting detection system per the manufacturer's instructions (GE Healthcare, Piscataway, NJ).

Proliferation assay

Proliferation was assessed by measurement of DNA content. After lentivirus infection, approximately 10^3 cells were plated in 96-well plates, and cells were fixed 2 and 6 days after infection. DNA was quantified using CyQuant Cell Proliferation kit (Invitrogen), and plates were read using the Spectramax M2 fluorescence microplate reader (Molecular Devices, Sunnyvale, CA) 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 using the Guava Nexin Kit (Guava Technologies, Hayward, CA) and compared to SCR. Briefly, lentivirus-infected cells were plated in triplicate in six-well plates. On days 2 and 6, floating and adherent cells were collected and washed once with cold PBS and once with 1X 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 using the Guava Personal Cytometer and analyzed using 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. 24-well BD BioCoat Matrigel Invasion membranes were rehydrated for 2 hours in a humidified tissue culture incubator (37°C, 5% CO_2), and 5.0×10^4 cells/ml of each liposarcoma cell line and ASCs were prepared in media without chemoattractant (DMEM HG/F12 + 1X P/S). Chemoattractant (DMEM HG/F12 + 10% FBS + 1X 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. Non-invading cells were removed from the upper surface of the membranes by vigorous scrubbing with a cotton-tipped swab. Cells that had invaded through the membranes were then stained with Diff-Quik stain and counted at 40X magnification in triplicate.

The percentage invasion was calculated by comparing the mean number of cells invading through the Matrigel insert membrane with the mean number migrating through the control insert membrane. The invasive index of the liposarcoma cell lines was calculated by dividing the percentage invasion of the test cell line (LPS141, DDLS8817, RC5397, and

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ML2308) by the percentage invasion of the control (ASCs). In the shRNA knockdown studies, the percentage invasion with TOP2A, PTK7, and CHEK1 shRNAs was compared to percentage invasion with SCR.

Results

Patient and Tumor Characteristics

In total, 140 patients with primary liposarcoma tumors were used to generate and evaluate the multi-gene 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 multi-gene predictor identified 588 genes whose expression correlated with DRFS (Supplementary Data). Increased DRFS was associated with 202 probe sets corresponding to 159 genes, while decreased DRFS was associated with 527 probe sets corresponding to 429 genes. Based upon expression of these 588 genes, a GRS was calculated for each patient's tumor. In the training set, patients with low GRS (below the median) had a 3-year DRFS of 88% compared with 43% for high GRS patients (on univariate analysis, $P<0.001$) (Supplementary Figure 1A). After subtype was adjusted for, the hazard ratio (HR) for high vs. 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% vs. 45% for high GRS patients ($P=0.001$) (Supplementary Figure 1B). The HR for high vs. low GRS was 4.42 (95% CI 1.26–15.55; $P=0.021$) after subtype was adjusted for.

To determine if a smaller subset of genes could accurately predict DRFS, the analysis was repeated with the number of probe sets in the predictor restricted to <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% vs. 45% for high GRS patients ($P<0.001$) (Figure 1A). When adjusted for subtype, the HR for high vs. low GRS was 1.54 (95% CI 1.09–2.18; $P=0.015$). In the test set, 3-year DRFS was 86% for low GRS patients vs. 44% for high GRS patients ($P=0.004$) (Figure 1B). The HR for high vs. low GRS was 1.53 (95% CI 1.00–2.35; $P=0.05$) when adjusted for subtype.

The accuracy of predicting DRFS was assessed by the concordance probability. In both training and test sets, the concordance probability was greater for GRS than for histologic subtype. For example, in the test set, concordance probability was 0.669 for subtype, 0.732 for GRS from the 588-gene predictor, and 0.721 for GRS from the 11-gene predictor. In both cases, concordance probability was highest when GRS was combined with subtype: in the test set, 0.742 for 588-gene GRS plus subtype and 0.726 for 11-gene GRS plus subtype.

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, CHEK1, TOP2A), spindle assembly checkpoint control (BUB1B, CDC20, MAD2L, PLK1), angiogenesis (VEGF), apoptosis (BAX, BIRC5,

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SOX4), tumor growth (KRAS, PLAU), and tumor invasion (CDKN2A, CXCL12, MMP14), amongst 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 ten 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 fifteen genes that were found in more than one of the top ten canonical pathways (Supplementary Table 2), as these genes may have a greater impact in metastasis of liposarcoma.

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. CHEK1 was chosen because it had one of the greatest marginal effects (univariate HR=8.24) and was found in five of the top ten canonical pathways. In addition, all three genes were significantly overexpressed in most of our liposarcoma cell lines when compared with ASCs.

Expression levels of TOP2A, PTK7, and CHEK1 were all strongly associated with outcome (Figure 2). For each gene, 3-year DRFS was significantly worse for patients with high-expressing tumors than for patients with low-expressing tumors: 45% vs. 91% for TOP2A, 49% vs. 87% for PTK7, and 49% vs. 87% for CHEK1 in univariate analysis (all $P<0.001$). After histologic subtype was adjusted for, expression of TOP2A (HR 1.55; 95% CI 1.04–2.29), PTK7 (HR 3.71; 95% CI 1.84–7.50), and CHEK1 (HR 4.39; 95% CI 1.37–14.06) each remained independent predictors of decreased DRFS.

Expression of TOP2A, PTK7, and CHEK1

In a comparison of gene expression in primary liposarcoma tumor samples versus normal fat using U133A Affymetrix gene arrays, TOP2A, PTK7, and 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 four liposarcoma cell lines for TOP2A (32-to 77-fold; all $P<0.05$) (Supplementary Figure 2A) and, to a lesser extent, for CHEK1 (2.4- to 3.0-fold; all $P<0.05$) (Supplementary Figure 2C). PTK7 expression was increased in LPS141, RC5397, and ML2308 cells (2.4- to 3.3-fold; all $P<0.05$), but not in DDLS8817 cells (Supplementary Figure 2B). Immunoblotting confirmed upregulation of TOP2A and CHEK1 protein expression (Supplementary Figure 2D).

shRNA Knockdown of TOP2A, PTK7, and CHEK1

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

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

Proliferation after shRNA Knockdown

shRNA knockdown of TOP2A, compared to 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 (Figure 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 Figure 4A, B). shRNA knockdown of CHEK1 with the CHEK1#4 shRNA compared to SCR decreased proliferation of DDLS8817 (71.8% ± 15.3%, $P<0.001$) and ML2308 (34.6% ± 14.3%, $P < 0.01$) but not ASCs (Figure 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 to SCR in DDLS8817 (43.4% vs. 13.6%, $P<0.001$) and in ML2308 (34.6% vs. 13.2%, $P<0.001$) (Figure 3B). Similarly, treatment with the PTK7#7 construct compared to SCR increased late apoptosis of LPS141 (29.3% vs. 9.9%, $P<0.001$), and treatment with the CHEK1#4 construct compared to SCR increased late apoptosis of DDLS8817 (34.2% vs. 10.9%) and ML2308 (24.7% vs. 14.1%) ($P < 0.001$) (Figure 3B). Similar increases in apoptosis were observed with TOP2A, PTK7, and CHEK1 knockdown using a second independent shRNA construct (Supplementary Figure 5). Knockdown of TOP2A, PTK7, and CHEK1 produced no difference in early apoptosis (day 2) in any of the liposarcoma cell lines tested, and no differences in early or late apoptosis in ASCs.

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 fold to 8.3 fold more invasive than the negative control (ASCs) ($P<0.001$) (Supplementary Figure 6).

For evaluating the effects of knockdown on invasiveness, we assessed invasiveness at two 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 $P<0.01$) (Figure 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 using a second shRNA construct (Supplementary Figure 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 multi-gene predictor, which uses expression of 588 genes to identify patients at low vs. high risk of metastasis. In external validation, the multi-gene predictor accurately predicted worse 3-year DRFS for those patients with high GRS tumors than for those with low GRS tumors with a hazard ratio 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 multi-gene

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predictor, the smaller gene predictor accurately segregated patients into high- and low-risk subgroups. Both predictors had higher concordance probability than did 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 multi-gene 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 end-point of disease-specific survival, whereas we used distant-recurrence–free survival.

Gene expression profiling can be useful in identifying potential therapeutic targets (2,16,17). Based on the association of TOP2A, PTK7, and CHEK1 with reduced DRFS, we conducted functional analyses of these genes. Expression of all three genes was elevated in primary liposarcomas and in most or all of our four liposarcoma cell lines. shRNA knockdown of these three genes confirmed their importance in liposarcoma cell lines. For all three genes, knockdown decreased cell proliferation and invasiveness and increased apoptosis. 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 chemotherapeutic 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 demonstrated 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 up-regulated 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 anti-apoptotic 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 up-regulated CHEK1.

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We have developed a multi-gene predictor that is more prognostic for DRFS than is 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 multi-gene predictor. Three genes from the multi-gene 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 multi-gene predictor may serve as candidates for targeted therapeutics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig 1A

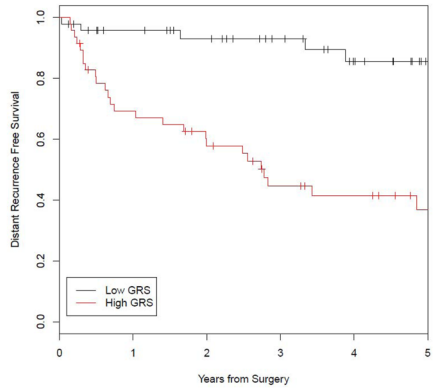

Fig 1B

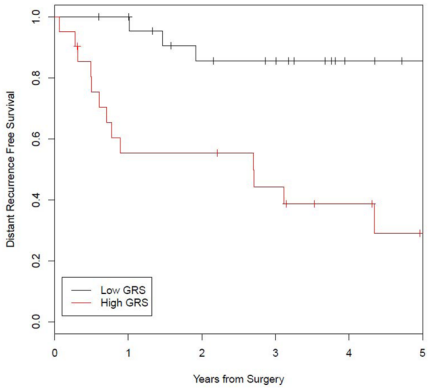

Figure 1.
Kaplan-Meir curves of distant-recurrence–free survival among patients with liposarcoma according to score in the 11-gene predictor, dichotomized at the median. A, patients in the training set. B, patients in the test set.

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Fig 2A

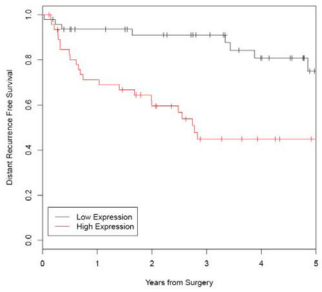

Fig 2B

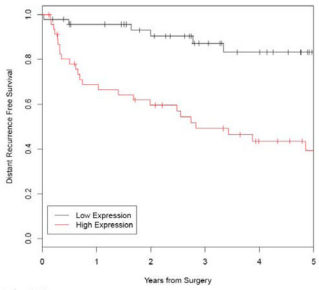

Fig 2C

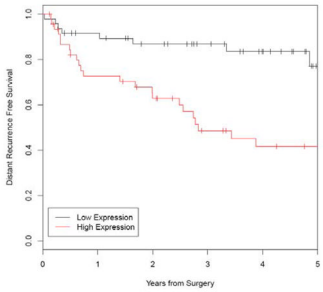

Figure 2.
Kaplan-Meir analysis of distant-recurrence–free survival in the training set according to gene expression. A, TOP2A expression; B, PTK7 expression; C, CHEK1 expression.

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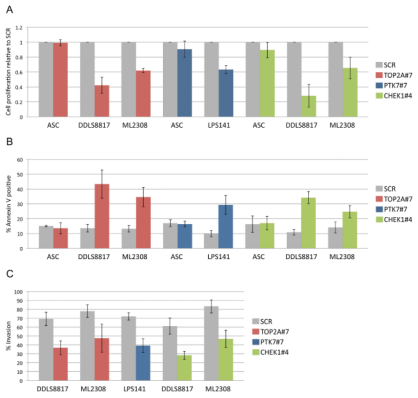

Figure 3.

Effects of TOP2A, PTK7, and CHEK2 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.

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Table 1

Characteristics of the 140 patients in the study.

Patient Characteristic	n	% of Total
Age (yr) [median (range)]	58 (27–90)	
Gender		
Female	59	42.1
Male	81	57.9
Histologic variant		
Well-differentiated	52	37.1
Dedifferentiated	39	27.9
Myxoid	17	12.1
Round cell	12	8.6
Pleomorphic	20	14.3
Primary site		
Lower extremity	62	44.3
Upper extremity	6	4.3
Trunk	4	2.9
Retroperitoneum	63	45.0
Thorax	5	3.5
Tumor depth		
Superficial	4	2.9
Deep	136	97.1
Tumor burden (cm) [median (range)]	17 (4.5–66)	
Margins		
Negative margins	88	62.9
Positive micro margins	46	32.8
Positive gross margins	6	4.3

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Table 2

Genes from the 11-gene predictor.

Gene Symbol	Gene Name	Function	Univariate Hazard Ratio*
ADH1B	Alcohol Dehydrogenase 1B	Ethanol oxidation	0.77
ADIPOQ	Adiponectin	Regulation of glucose and lipid metabolism	0.78
ANK2	Ankyrin 2	Cell motility and proliferation	0.53
GPD1	Glycerol-3-phosphate Dehydrogenase 1	Glycolytic pathway enzyme	0.61
NTRK2	Neurotrophic Tyrosine Kinase Receptor Type 2	Signal transduction and cellular differentiation	0.74
PLIN	Perilipin	Inhibition of lipolysis	0.72
RBP4	Retinol Binding Protein 4	Carrier for retinol in the blood	0.77
MMP14	Matrix Metallopeptidase 14	Breakdown of extracellular matrix	2.84
PTK7	Protein Tyrosine Kinase 7	Extracellular signal transduction and cell adhesion	4.16
RAB23	RAB23, Member RAS Oncogene Family	GTPase-mediated signal transduction and intracellular protein transport	1.85
TOP2A	Topoisomerase II Alpha	Alteration of DNA topologic state during transcription	1.70, 1.85†

*Hazard ratios >1 imply that increased expression is associated with shorter distant recurrence–free survival.

†TOP2A has two hazard ratios that correspond to two different probe sets.

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Table 3

Top 10 Canonical Pathways from Ingenuity Pathway Analysis.

Canonical Pathway	*P*-value	Genes in Pathway*
Mitotic Roles of Polo-like Kinase	2.9×10^{-7}	12
Pyruvate Metabolism	3.4×10^{-4}	9
G2/M DNA Damage Checkpoint Regulation	3.4×10^{-4}	7
p53 Signaling	9.5×10^{-4}	10
Aryl Hydrocarbon Receptor Signaling	1.1×10^{-3}	13
Factors Promoting Cardiogenesis in Vertebrates	7.1×10^{-3}	8
Role of BRCA1 in DNA Damage Response	7.9×10^{-3}	6
Glycolysis - Gluconeogenesis	8.2×10^{-3}	8
ATM Signaling	8.7×10^{-3}	6
Mechanism of Viral Exit from Host Cells	1.0×10^{-2}	5

*Number of genes from the 588-gene predictor