Doxorubicin Resistance in a Novel In vitro Model of Human Pleomorphic Liposarcoma Associated with Alternative Lengthening of Telomeres

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Abstract

Soft tissue sarcomas are a diverse set of fatal human tumors where few agents have demonstrable clinical efficacy, with the standard therapeutic combination of doxorubicin and ifosfamide showing only a 25% to 30% response rate in large multi-institutional trials. Although liposarcomas are the most common histologic form of adult soft tissue sarcomas, research in this area is severely hampered by the lack of experimentally tractable in vitro model systems. To this end, here we describe a novel in vitro model for human pleomorphic liposarcoma. The cell line (LS2) is derived from a pleomorphic liposarcoma that uses the alternative lengthening of telomeres (ALT) mechanism of telomere maintenance, which may be important in modulating the response of this tumor type to DNA-damaging agents. We present detailed baseline molecular and genomic data, including genome-wide copy number and transcriptome profiles, for this model compared with its parental tumor and a panel of liposarcomas covering multiple histologies. The model has retained essentially all of the detectable alterations in copy number that are seen in the parental tumor, and shows molecular karyotypic and expression profiles consistent with pleomorphic liposarcomas. We also show the utility of this model, together with two additional human liposarcoma cell lines, to investigate the relationship between topoisomerase 2A expression and the sensitivity of ALT-positive liposarcomas to doxorubicin. This model, together with its associated baseline data, provides a powerful new tool to develop treatments for this clinically poorly tractable tumor and to investigate the contribution that ALT makes to modulating sensitivity to doxorubicin. Mol Cancer Ther; 9(3): 682–92. ©2010 AACR.

Introduction

Sarcomas are rare mesenchymal malignancies characterized by >100 different histologies. Among this diverse group of cancers, liposarcomas comprise one of the most common histopathologic types in adults over 55 years of age. These adipocytic tumors show heterogeneous histologies, including well-differentiated, dedifferentiated, pleomorphic, and myxoid/round cell types. The well-differentiated liposarcomas, also called atypical lipomatous tumors, can be further subdivided into four commonly recognized subgroups: adipocytic, inflammatory, sclerosing, and spindle cell. The spindle cell morphology is believed to represent a higher-grade version of well-differentiated liposarcomas. As suggested by their names, both the dedifferentiated and pleomorphic liposarcomas are considered higher-grade malignancies. Myxoid and round cell tumors contain a translocation fusing the CHOP gene on chromosome 12 to either FUS on chromosome 16 in 90% of the cases or EWS on chromosome 22 in the remaining 10% of the cases. In contrast, the other histologic variants of liposarcoma are characterized by complex numerical and structural karyotypic changes, including the presence of supernumerary chromosomes carrying material from chromosomes 12q and 1q. Expression profiles of the various histologic subtypes of liposarcomas have been generated, and not surprisingly, well-differentiated liposarcomas resemble mature adipocytes, whereas the higher-grade tumors show a progressive loss of the adipose signature (1, 2).

Telomeres are specialized structures composed of hexanucleotide DNA repeats and associated proteins...
that provide stability to chromosome ends. Maintenance of telomeres confers replicative immortality (3) and is a fundamental characteristic of most cancer cells (4). The majority of neoplasias achieve telomere maintenance via increased activity of a specialized reverse transcriptase, telomerase (5), which uses an RNA template molecule to add telomeric DNA sequences de novo onto chromosome ends (6). Telomerase-independent mechanisms for telomere maintenance have also been described and are collectively termed alternative lengthening of telomeres (ALT; ref. 7). ALT uses recombination-based pathways to elongate telomeric arrays.

We have previously characterized telomere maintenance in liposarcomas and found roughly equal frequency of telomerase and ALT activity (~25% each), whereas approximately half of the tumors did not have characteristics of either pathway (8). Similar results were obtained by Costa et al. (9). Recently, using a PCR-based assay to measure recombination at subtelomeric regions, which is elevated in ALT-positive cells and tumors, Jeyapalan et al. (10) suggested that some tumors in the third category (e.g., without characteristics of ALT or telomerase) may have ALT activated without exhibiting all the characteristics of the pathway. ALT-positive liposarcomas have the worst prognosis followed by telomerase-positive tumors, whereas the best prognosis was associated with tumors devoid of characteristics of either pathway (9). Using whole-genome profiling, we identified deletion of chromosome 1q as the most frequent change in ALT-positive tumors, whereas this imbalance was only rarely observed in telomerase-positive tumors (11). In contrast, amplification of chromosome 12q was underrepresented in ALT-positive tumors but observed frequently in the non-ALT tumors. We hypothesize that alterations such as those associated with the mechanism of telomere maintenance may underlie the differences in patient outcome that have been observed in liposarcomas.

The ability to test the role of candidate genes on tumor cell phenotypes has been hampered by the histologic heterogeneity and limited availability of cell lines derived from liposarcomas (12–17). Here, we describe a new cell line, LS2, derived from an ALT-positive pleomorphic liposarcoma. The LS2 cell line carries the chromosome 1q deletion and several chromosome anomalies observed in pleomorphic liposarcomas, making this cell line a useful tool to dissect pathways critical for the more aggressive phenotype of ALT-positive liposarcomas. We also report extensive molecular genetic characterization of both the LS2 cell line and its tumor of origin. To our knowledge, this is the only liposarcoma cell line to date for which extensive copy number and expression information is published. Because detailed molecular information on the tumor is available for baseline comparison, the conservation of genetic alterations present in the LS2 cell line can be validated rapidly.

Materials and Methods

Cell Culture

Collection of liposarcomas for studying mechanisms for maintaining telomeres and development of cell lines was done using an Institutional Review Board-reviewed protocol at Fox Chase Cancer Center. The LS2 cell line was derived from a pleomorphic liposarcoma; it was placed in culture after mechanical disruption. LS2 is maintained in RPMI 1640 + Glutamax (Invitrogen) supplemented with 20% fetal bovine serum (FBS; Invitrogen), MEM vitamin mixture (BioWhittaker), ITES (Lonza), penicillin (100 units/mL)–streptomycin (100 μg/mL)–l-glutamine (0.29 mg/mL) mixture (Invitrogen), 1 mmol/L sodium pyruvate (CellGro), and MEM Eagle nonessential amino acid solution (Lonza) with 5% CO2. The LiSa-2 cell line, derived from a poorly differentiated, pleomorphic liposarcoma (12), was provided by Dr. W. Chow (City of Hope, Duarte, CA) and is maintained in DMEM (Invitrogen) supplemented with 10% FBS, 25 mmol/L HEPES (pH 7.3), and penicillin (100 units/mL)–streptomycin (100 μg/mL)–l-glutamine (0.29 mg/mL) mixture with 5% CO2. The SW872 cell line was obtained from the American Type Culture Collection (ATCC) and is maintained as suggested by the ATCC in the absence of CO2 and in Leibovitz’s L15 medium (Invitrogen) supplemented with 10% FBS, 0.29 mg/mL l-glutamine, and 0.1 μg/mL Normocin (Invivogen). The HeLa cell line was maintained in DMEM supplemented with 10% FBS and penicillin (100 units/mL)–streptomycin (100 μg/mL)–l-glutamine (0.29 mg/mL) mixture with 5% CO2.

DNA fingerprints were obtained for T27, the LS2 cell line derived from T27, and the LiSa-2 cell line using the AmpFISTR Identifier PCR Amplification kit (Applied Biosystems) as recommended by the manufacturer. The kit amplifies the amelogenin gender-determining marker and 15 tetranucleotide repeat loci in a single PCR amplification. This combination of markers is consistent with worldwide database recommendations for identity testing. Allele calls were made from peak plots by comparing peaks with known fragment sizes using GeneMapper 4.0 (Applied Biosystems).

Telomere Repeat Amplification Protocol

The PCR-based telomere repeat amplification protocol (TRAP) assay of telomerase enzyme activity was carried out as described previously (18, 19). Briefly, cellular extracts were prepared by homogenizing cells in either CHAPS extraction buffer or Buffer C (20 mmol/L HEPES (pH 7.9), 420 mmol/L KCl, 5 mmol/L MgCl2, 25% glycerol, 0.1 mmol/L EDTA, 0.2% NP40). Total protein concentrations were determined using the detergent-compatible protein assay (Bio-Rad). Equivalent amounts of extract corresponding to 0.1 to 1 μg of total protein were used for each reaction. At least three independent extracts were made for each cell line. At least three independent TRAP assays were done for each extract.

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Mol Cancer Ther; 9(3) March 2010
683
Reverse Transcription-PCR-Based Detection of Telomerase Components

Detection of hTERT message was done as described below in Taqman assays using the Hs00162669_m1 assay (Applied Biosystems). Expression level was determined using the ΔΔCt method, normalized to the level of 18S rRNA expression (assay 4308329; Applied Biosystems), and quantified relative to HeLa hTERT message levels. To detect transcripts encoding the hTR component of telomerase, 2.5 and 0.5 ng of total RNA were transcribed into cDNA as described for the Taqman assays in the presence and absence of reverse transcriptase. The cDNA was then subjected to PCR using primers designed to amplify the telomerase template RNA (5′-CTAACCACTGAGGCGCTA-3′) and (5′-GGCGAACGGGCCAGCAGCGTACATT-3′). Reactions were carried out in a commercially available buffer containing 15 mmol/L MgCl2 (Applied Biosystems). Reactions were incubated at 94°C for 1 min and then subjected to the following for 30 cycles: 94°C for 30 s, 65°C for 30 s, and 72°C for 45 s. Products were visualized on 6% acrylamide gels.

Immunofluorescence Detection of ALT-Associated PML Nuclear Bodies

Following pre-extraction in low-salt buffer and fixation in 3.7% formaldehyde, frozen tissue sections or cultured cells were subjected to detection of TRF2 using a mouse monoclonal antibody raised against the full-length human TRF2 protein (Imgenex) or a rabbit polyclonal antibody raised against the NH2-terminal acidic domain of hTRF1 (20) and the PML protein using a goat polyclonal antibody (N-19; Santa Cruz Biotechnology). A series of 0.5-μm sections were collected for five to seven fields for each sample. Three-dimensional reconstruction and quantitation of ALT-associated PML nuclear bodies (APB) in individual nuclei within each field was carried out with MetaMorph image analysis software (Universal Imaging/Molecular Devices) using the measured colocalization function. These analyses are described in detail elsewhere (8). Immunofluorescent analysis of the LS2 and LiSa-2 cell lines was carried out at least thrice over a period spanning several months.

Expression Profiling

Total RNA was isolated using Trizol reagent and purified using RNaseasy spin columns (Qiagen). RNA purity was determined by analysis using a NanoDrop spectrophotometer. The quality of the RNA was determined using the Agilent 2100 Bioanalyzer. The RNA was then processed for hybridization to the U133 Plus 2.0 arrays (Affymetrix) as follows. The Message II Biotin Enhanced Single Round aRNA Amplification kit (Ambion) was used to synthesize cDNAs using 1 μg of total RNA and protocols provided by the manufacturer. Synthesis of biotin-labeled RNA was carried out using the Ambion kit as above with T7 RNA polymerase in the presence of a biotinylated nucleotide analogue/ribonucleotide mix. The aRNA yield was determined by spectrophotometric analysis at 260 nm, and size distribution of aRNA was determined with the Agilent 2100 Bioanalyzer. A fragmentation reaction resulted in a distribution of 35- to 200-nucleotide aRNA fragments (reaction mix provided by Ambion). Hybridization of the labeled aRNA to the U133 Plus 2.0 arrays was carried out in the presence of herring sperm DNA and probe array controls provided by the manufacturer. The probe array was stained and washed on station and scanned using the Affymetrix GeneChip Scanner 3000. The expression profiles of the LS2 cell line and the originating tumor were done in triplicate.

In addition to the tumor (T27*) from which the LS2 cell line was derived, we analyzed 30 independent human liposarcoma tumor samples for expression profiling. This group consisted of samples that had a clear diagnosis of pleomorphic, well-differentiated, myxoid, or dedifferentiated liposarcoma, thus paralleling the work of Matushansky et al. (1). Specifically, these samples consisted of 6 pleomorphic samples [T2*, T31R2*, a recurrence of T31R1*], T33, T34, T35, and T36, 3 dedifferentiated tumors (T15*, T29*, and T37), 5 myxoid-type tumors [T7*, T17*, T20*, T23*, and T39 (round cell type)], and 16 well-differentiated tumors [T6*, T10*, T19*, T21*, T31R1* (spindle cell), T32R1* (spindle cell), T32R2* (a spindle cell recurrence of T32R1*), T40, T41, T42, T43, T44, T45, T46, and T47]. The tumors marked with an asterisk have been characterized previously to determine which telomere maintenance mechanism is active and interrogated for copy number changes using whole-genome profiling (8, 11).

Quality control was done on MAS5 data using Affymetrix Expression Console software. All analyses were done using Bioconductor packages (8) in the R environment. Expression summary values were extracted using robust multiarray average (21). A representative probe set for each of the genes reported in Table 1 of Matushansky et al. (1) was chosen. The log-space expression data for this gene list were visualized using a heat map after median centering and hierarchical clustering using cosine similarity and average linkage, using a custom R script. Both the whole-genome (see below) and expression profiling data sets are available on the Gene Expression Omnibus database.9

Cytogenetic Analysis

Cell cultures of high mitotic index were exposed to colcemid (0.1 μg/mL; Life Technologies) for 1 h at 37°C and harvested according to routine cytogenetic protocols. For the construction of the LS2 molecular karyotype, multiplex fluorescence in situ hybridization (M-FISH; Multicolor-FISH; Metasystems) was combined with G-banding and inverted 4′,6-diamidino-2-phenylindole banding.

G-banding was done after trypsin (Life Technologies) and Giemsa (BDH) staining. Multicolor M-FISH was done according to the manufacturer’s protocols. Conventional cytogenetic analyses were done using the Ikaros software, whereas molecular cytogenetic analyses were done by the aid of Isis software (Metasystems). Both cytogenetic work stations were equipped with Zeiss microscopes. A total of 20 metaphase nuclei from two independent harvests were analyzed.

Whole-Genome Profiling
Analysis of copy number changes was carried out as described (11) using the Affymetrix GeneChip single-nucleotide polymorphism 100K mapping array. For each sample, 250 ng of purified DNA were digested with XhoI, and the resulting fragments were ligated to adaptor sequences using T4 DNA ligase. PCR-amplified product was then fragmented by DNaseI, denatured to generate single-stranded product, and end-labeled by terminal deoxynucleotidyl transferase. The labeled probe was hybridized to GeneChip arrays, washed, stained, and visualized using an Affymetrix GeneChip Scanner 3000. The intensity of probe signals following hybridization to the GeneChip was determined using Affymetrix GeneChip Operating Software v1.2. The data were analyzed as described by Johnson et al. (11)

Telomere Length Analysis
Genomic DNA was isolated by SDS lysis from the LS2 and LiSa-2 cell lines at several passages spanning 5 mo for LS2 and 10 mo for LiSa-2. Genomic DNA was also prepared from the SW872 cell line, which is available from the ATCC. Telomere length was determined by Southern analysis of terminal restriction fragments using the TeloTAGGG kit (Roche).

Quantitative Real-time PCR
To determine expression levels of targets in the cell lines, RNA was isolated using the RNeasy kit (Qiagen). For analysis of expression levels in tumors, RNA was isolated using Trizol reagent. The RNA was then passed over RNeasy columns to remove impurities. We prepared cDNA using 100 ng of input RNA and the High Capacity Reverse Transcriptase kit as recommended by the manufacturer (Applied Biosystems). Taqman assays were carried out in triplicate using the 18S rRNA assay (4308329) as the endogenous control. For topoisomerase 2A, the expression level in cell lines was compared with RNA isolated from adult adipocyte stem cells, whereas the expression in tumors was compared with RNA isolated from adipose tissue (Ambion) using the Hs00172214_m1 assay (Applied Biosystems). Expression levels of TOP2A were calculated using the standard curve method with HeLa RNA used to generate the standard curve.

Results
LS2, a New Cell Line Derived from a Pleomorphic Liposarcoma, Uses ALT for Telomere Maintenance
The LS2 cell line was derived from a high-grade pleomorphic liposarcoma from a 68-year-old African American female who noted a lump on her right forearm. Ultrasound revealed a complex mass measuring 4.5 × 3.4 × 2.1 cm, and magnetic resonance imaging revealed a 4.3 × 2.8 × 2.5 cm mass that raised concern for sarcoma. An incisonal biopsy 3 months later was suggestive of a high-grade liposarcoma. The patient subsequently underwent definitive resection. Pathology revealed a 3.5 × 2.5 × 2 cm mass, having light tan rubbery bulging glistening features, consistent with a high-grade pleomorphic liposarcoma invading the skeletal muscle. There was no evidence of any angiolyphatic invasion. Surgical margins were negative. The patient was followed, and on a computed tomography scan done 1 year later, multiple pulmonary nodules were seen with a subsequent biopsy confirming metastatic disease. The patient was started on investigational therapy with trabectedin in combination with docetaxel (22) for eight cycles that was discontinued due to progression of disease. She then received three cycles of liposomal doxorubicin that was discontinued secondary to progressive disease. The patient died of her disease 5 months later.

Fresh tumor tissue was obtained at the time of the initial resection, and cells were placed in culture after mechanical disruption. Continuously growing cultures have attained more than 122 passages and 200 population doublings and are now termed the LS2 cell line. Telomere maintenance was first assessed at a relatively early passage and periodically thereafter. No passages of LS2 tested have telomerase activity as assessed by the TRAP assay (Fig. 1A). The tumor from which LS2 was derived was also found to be telomerase negative (11). Similarly, the established LiSa-2 cell line does not have telomerase activity, as shown by the TRAP assay, whereas the SW872 cell line exhibits telomerase activity (Fig. 1A). Consistent with the absence of telomerase enzyme activity, LS2 cells do not express mRNA for the catalytic subunit of telomerase, hTERT, despite the presence of the RNA template component, hTR, both as assessed by reverse transcription-PCR (Fig. 1B and C). In contrast, the LiSa-2 cell line is negative for telomerase activity when evaluated by the TRAP assay yet expresses both hTERT and hTR. As expected, the telomerase-positive SW872 cell line expresses both basic components of the telomerase holoenzyme (Fig. 1B and C). ALT-positive cells and tumors are characterized by long heterogeneously sized telomeres. Southern analysis of terminal restriction fragments confirmed the presence of ALT-like telomeres in the LS2 and LiSa-2 cell lines (Fig. 1D), as well as in the tumor from which the LS2 cell line was established. As expected, telomere length in the telomerase-positive SW872 cell line was significantly shorter than in LS2 or LiSa-2, being <3 kb overall. Telomere length was assessed...
at different times and remained stable over several months in culture. Indirect immunofluorescence analysis showed the presence of APBs (wherein the telomeric components colocalize with PML nuclear bodies) in the LS2 and LiSa-2 cell lines as well as in sections from T27, the tumor from which LS2 was derived (Fig. 1E). Minor differences in the frequency of APBs in the tumor T27 and its derivative LS2 cell line likely reflect different growth environments (in vivo versus in vitro) and minor differences in the genetic makeup of LS2 and T27 (see below).

Figure 1. Analysis of telomere maintenance mechanism. A, TRAP assay of ALT-positive LS2 and LiSa-2 cell lines and the telomerase-positive SW872 cell line. HeLa extract was mixed with extracts in the indicated lanes to control for the presence of telomerase inhibitors in the extracts, whereas treatment of the extracts with RNase inhibited formation of telomerase products. LS2 was assessed at early (PD2) and late passage (PD172). For LiSa-2, which was established in a different laboratory (12), extracts were prepared at a designated time (passage 0) and following 10 mo in culture (passage 66). B, hTERT expression level was assessed by Taqman assay in three liposarcoma-derived cell lines: LS2, LiSa-2, and SW872. Expression levels are displayed in comparison with those of hTERT in HeLa cells and were normalized to the expression level of 18S rRNA. C, the telomerase template RNA hTR is expressed in all three liposarcoma cell lines. Total RNA was analyzed by reverse transcription-PCR in the presence (+RT) and absence (−RT) of reverse transcriptase. M, molecular weight markers of the indicated size in base pairs. D, Southern analysis of telomere length in the original tumor (T27), the LS2 cell line, and the LiSa-2 cell line. Telomere length in the LS2 and LiSa-2 cell lines was assessed over several months in culture. For LS2, DNA was prepared at the indicated passages, spanning 5 mo in culture, whereas for LiSa-2 DNA was prepared at the indicated passages spanning 10 mo in culture. Note the presence of long heterogeneous telomeres in all three samples, in contrast to the shorter telomeres present in telomerase-positive SW872 cells. E, analysis of ALT-associated PML bodies in the LS2 cell line, the original tumor (T27), the LiSa-2 cell line, and the SW872 cell line. Indirect immunofluorescence shows colocalization of the telomeres (red; either hTRF2 or hTRF1) with the PML nuclear bodies (green). DNA is stained with 4′,6-diamidino-2-phenylindole (blue).
The SW872 cell line did not contain APBs (Fig. 1E) and, as predicted based on telomere length (Fig. 1D), had very weak staining of telomeres. Based on telomerase negativity, heterogeneous telomere length, and APB positivity, we classify LS2 and LiSa-2 as ALT-positive liposarcoma cell lines, whereas the SW872 cell line is telomerase positive. Both of the telomere maintenance characteristics (telomerase activity and the absence or presence of APBs) were monitored at regular intervals and have been retained throughout the culture of the LS2, SW872, and LiSa-2 cell lines (Fig. 1A and D).

**Whole-Genome Profiling Shows That LS2 Is Most Closely Related to the Tumor from Which It Is Derived**

Whole-genome profiling of DNA isolated from LS2 showed that copy number changes present in the original tumor are retained in the cell line (Fig. 2). The LS2 cell line is notably more similar to the tumor from which it was derived than it is to other pleomorphic liposarcomas or to liposarcomas of other histologies (e.g., myxoid, dedifferentiated, or well differentiated; Fig. 2; Supplementary Fig. S1). The only pronounced differences between the LS2 cell line and the original tumor are on chromosome 14, where the LS2 cell line contains a deletion of \( \sim 7.5 \text{ Mb} \) spanning the region Chr.14q24.3-q31.2 and amplification of most of Chr.5q (Fig. 2), neither of which is present in the original lesion.

There are several alterations in copy number spanning >2.5 Mb of DNA that are shared between LS2 and the original tumor. These include the chromosome 1 deletion, Chr.1q32.2-q44, which we have previously reported to be associated with ALT-positive liposarcomas (Fig. 2; ref. 11). Other changes shared between the tumor and the LS2 cell line include deletion of Chr.2q36.3-q37.3, amplification of Chr.20p13-p12.3, amplification of chromosome 5p, and amplification of large portions of chromosomes 9q, 13q, and 18q.

**Cytogenetic Analysis of LS2**

Similar to several ALT-positive human tumor cell lines (23), the near-tetraploid LS2 karyotype is characterized by highly increased breakage/fusion/bridge cycle-induced structural instability. This was verified by the mitotic presence of numerous telomere rearrangements, inverted duplications, and random dicentric chromosome formations. Furthermore, the LS2 karyotype displays high frequencies of neoacrocentric and minute chromosomes, which were recently proposed to be a hallmark of the ALT chromosomal constitution (23). Although there are different coexisting subclones in the LS2 cultures and the chromosome number deviates between 79 and 183 (with a rate of whole-genome duplication estimated between 10% and 12% of dividing cells), all LS2 subclones seemed to have a monoclonal origin because they shared several characteristic structural chromosomal anomalies. We analyzed a major subclone of these cells by M-FISH (Fig. 3). A detailed interpretation

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**Figure 2.** A plot of nucleotide position on the X axis (left to right ascending nucleotide position from nucleotide 1 at the telomere of the p arm to the q arm telomere) versus copy number (value from GeneChip Operating Software; diploid = 2) on the Y axis (ascending copy number from bottom to top) for each probe, shown separately for each autosome comparing the LS2 cell line and the original tumor, T27. Green asterisks, conserved copy number changes between T27 and the derived LS2 cell line; red asterisks, differences.
of the representative karyotype of this LS2 subclone, according to the International System for Cytogenetic Nomenclature (24), is presented in Supplementary Data. Based on this analysis, the molecular karyotype of LS2 shares several chromosome abnormalities with those previously reported in the few cases of pleomorphic liposarcomas that have been cytogenetically characterized. These are deletions of 1q, 2p, and 3p and rearrangements of both arms of chromosomes 19 and 20 (25, 26). Notably, several but not all of the imbalances that have been detected by whole-genome profiling could be recapitulated using M-FISH. Confirmed imbalances involve the chromosome 1q deletion and losses of genomic material from 2p, 2q, and 3p. Discrepancies between the two methods concerned amplification of 5p, 13q, and 18q that were not evident in the subclone analyzed by M-FISH. This divergence may be attributed to the extensive chromosomal instability and karyotypic heterogeneity of the LS2 cell line. Taken together, the above results indicate that the molecular cytogenetic profile of LS2 cells follows the characteristics of the ALT pathway but also exerts some of the recurrent features observed in pleomorphic liposarcomas.

LS2 Has an Expression Profile Consistent with Pleomorphic Liposarcoma

Expression analysis of liposarcomas has been carried out previously by several groups (1, 2, 27). A recent report found that the expression profiles of liposarcomas can be clustered based on histology and suggested a differentiation-based classification for these tumors (1). We carried out a supervised analysis of the expression pattern of LS2 and a panel of liposarcomas of various histologies using the gene list identified as being specific for adipogenesis (Table 1 in ref. 1). LS2 clustered with pleomorphic liposarcomas in this analysis, indicating that it retains the expression signature characteristic of this subtype of liposarcoma (Fig. 4). Key characteristics include loss of expression of genes characteristic of adipogenesis, such as lipoprotein lipase, adiponectin, and leptin. Although LS2 retains an expression pattern that is overall more closely aligned with pleomorphic liposarcomas.
than with other subtypes of liposarcoma, with respect to this gene list it is not identical to the tumor from which it was derived. This discordance might reflect subtle genetic or epigenetic changes resulting from culturing LS2 cells ex vivo. Importantly, LS2 clusters closely with the original tumor when the gene list used in a supervised analysis is the Cell Division Gene Ontology category composed of markers of proliferation (GO:0051301; Supplementary Fig. S2), indicating that, as expected, many genes are similarly regulated in LS2 and the original tumor.

**LS2 Sensitivity to Doxorubicin Is Correlated to TOP2A Expression Levels**

To assess the usefulness of LS2 as a surrogate experimental model for tumor behavior, we determined the sensitivity of LS2 to doxorubicin (Fig. 5A), which is commonly used in the treatment of these malignancies. Doxorubicin inhibits the activity of topoisomerases, and drug sensitivity has been correlated with the expression levels of the topoisomerase 2A gene (28). For comparison, the sensitivity of two other liposarcoma-derived cell lines was also determined. As noted above, the LS2 and LiSa-2 cell lines are ALT positive, whereas the SW872 cell line is telomerase positive (Fig. 1). The SW872 cell line was the most sensitive to doxorubicin followed by the LS2 cell line. The LiSa-2 cell line was the least sensitive to doxorubicin, with the cells retaining 20% viability at 1 μmol/L. As expected, sensitivity to doxorubicin correlated with expression levels of TOP2A as determined by quantitative real-time PCR; SW872 had the lowest expression level of TOP2A, whereas LiSa-2 had the highest expression level of this gene (Fig. 5B).

The expression level of TOP2A in the tumor from which LS2 was derived was also determined and, compared with the results obtained from an additional cohort of seven pleomorphic liposarcomas, was also determined.
TOP2A expression in the T27 tumor, from which the LS2 cell line was derived, is among the highest of all the tumors assayed (Fig. 5C). This is consistent with the lack of response to liposomal doxorubicin observed in the patient. Further analysis of the levels of TOP2A expression in well-differentiated liposarcomas indicates that, as a general rule, TOP2A expression is lower in these tumors than in the pleomorphic liposarcomas (Fig. 5C).

Discussion

Telomerase-independent mechanisms of telomere maintenance, such as ALT, provide an alternative route whereby transformed cells may overcome the growth limitation imposed by critically short telomeres (29). In addition, tumors using ALT for telomere maintenance should be refractory to treatment targeting telomerase, a strategy currently being tested in clinical trials (30, 31). Although a minority of human epithelial carcinomas have characteristics consistent with ALT utilization, ALT has been shown with relatively high frequency in osteosarcomas (32), glioblastoma multiforme (33), and other malignancies of mesenchymal origin. Indeed, ALT is used as frequently as telomerase in soft tissue sarcomas (34), including the most common subtype, liposarcoma (8, 9). Efficacious treatment remains elusive for liposarcoma, however, perhaps a consequence of the high frequency of ALT utilization for telomere maintenance. The rarity of liposarcoma tumors has hampered the identification of mutations that contribute both to their development and to activation of the ALT mechanism. The ability to mechanistically explore these processes has likewise been limited by the corresponding rarity of cell lines.

Here, we describe the establishment of a new cell line derived from a pleomorphic liposarcoma. We believe that LS2 will serve as a potentially important model for ALT-positive liposarcomas, the prognosis of which is poorest for ALT positive when categorizing based on the telomere maintenance mechanism present in the sarcoma (9). The utility of LS2 is enhanced by our detailed genome-wide molecular characterization of both the cell line and its original tumor. The LS2 cell line retains the majority of DNA copy number changes present in the original tumor and has an expression profile consistent with pleomorphic liposarcomas. Consequently, LS2 represents an important and novel experimental tool that might be used to test hypotheses aimed at understanding the development of liposarcomas. In addition, the importance of the chromosome 1q deletion, which is characteristic of ALT (11) and is present in both the tumor and LS2 cell line, in regulation of ALT and sarcomagenesis can be tested in this model. Thus, LS2 will help us better understand not only the development of liposarcomas but also the pathways underlying the ALT mechanism, thereby revealing new targets for treatment of several clinically relevant malignancies that use recombination-based maintenance of telomeres.

According to Antonescu (35) two thirds of soft tissue sarcomas lack a recurrent genetic signature and are characterized by complex karyotypes with numerous structural and numerical chromosome anomalies. Most of the adult spindle cell and pleomorphic sarcomas belong to this group. Despite such complexity, however, the karyotype of the LS2 cell line shares some recurrent rearrangements with the reported karyotypes of pleomorphic liposarcomas, including deletions in the long arm of chromosome 1 and deletions of 2p and monosomies 13, 14, 16, and 22 (25, 26, 36). The role of these chromosomal changes in tumor phenotype can be determined using the LS2 cell line model system. Cytogenetic characterization
of cell lines derived from well-differentiated (GOT3; ref. 17), dedifferentiated (FU-DDLS-1; ref. 14), and retroperitoneal (HTLS; ref. 15) liposarcomas has been described. Comparison with the original tumor is only available for the GOT3 cell line (17). Both the GOT3 and FU-DDLS-1 contain the Chr.1q2 amplon, which is not present in the LS2 cell line (14, 17). In contrast, neither cell line contains the Chr.1q deletion characteristic of ALT-positive liposarcomas, which is present in both LS2 and the tumor T27 from which it was derived.

Chemotherapy regimens for treating liposarcoma have had limited efficacy. Thus, new targets are needed. The LS2 cell line will significantly add to the cell-based models currently available for testing new compounds with potential therapeutic benefit for liposarcomas. The LS14 cell line, derived from a metastatic liposarcoma, is more resistant to doxorubicin than the SW872 cell line (37). We find SW872 to be the most sensitive of the three liposarcoma cell lines tested in the study described here. Importantly, this particular cell line, LS2, not only replicates the expected biological findings but also recapitulates the clinical experience with limited sensitivity to doxorubicin observed in the original tumor, T27. LS2 therefore presents a good model system in which to investigate the importance of candidate genes on activation of ALT for telomere maintenance and on ALT-associated tumor phenotypes, such as poor patient prognosis in liposarcomas (9).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Doxorubicin Resistance in a Novel *In vitro* Model of Human Pleomorphic Liposarcoma Associated with Alternative Lengthening of Telomeres

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