Published in final edited form as:

J Surg Oncol. 2015 May ; 111(6): 669–682. doi:10.1002/jso.23860.

Characterization of a Novel Radiation-Induced Sarcoma Cell Line

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Abstract

Background—Radiation-induced sarcoma (RIS) is a potential complication of cancer treatment. No widely available cell line models exist to facilitate studies of RIS.

Methods—We derived a spontaneously immortalized primary human cell line, UACC-SARC1, from a RIS.

Results—Short tandem repeat (STR) profiling of UACC-SARC1 was virtually identical to its parental tumor. Immunohistochemistry (IHC) analysis of the tumor and immunocytochemistry (ICC) analysis of UACC-SARC1 revealed shared expression of vimentin, osteonectin, CD68, Ki67 and PTEN but tumor-restricted expression of the histiocyte markers α 1-antitrypsin and α 1-antichymotrypsin. Karyotyping of the tumor demonstrated aneuploidy. Comparative genomic hybridization (CGH) provided direct genetic comparison between the tumor and UACC-SARC1. Sequencing of 740 mutation hotspots revealed no mutations in UACC-SARC1 nor in the tumor. NOD/SCID gamma mouse xenografts demonstrated tumor formation and metastasis. Clonogenicity assays demonstrated that 90% of single cells produced viable colonies. NOD/SCID gamma mice produced useful patient-derived xenografts for orthotopic or metastatic models.

Conclusion—Our novel RIS strain constitutes a useful tool for pre-clinical studies of this rare, aggressive disease. UACC-SARC1 is an aneuploid cell line with complex genomics lacking common oncogenes or tumor suppressor genes as drivers of its biology. The UACC-SARC1 cell line will enable further studies of the drivers of RIS.

Synopsis—We derived a spontaneously immortalized primary human cell line, UACC-SARC1, from a radiation-induced sarcoma (RIS). Our novel RIS cell line constitutes a useful tool for pre-clinical studies of this rare, aggressive disease.

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Declaration of competing interest: Julie Lang (Genomic Health - speaker bureau). Lee Cranmer (Threshold Pharmaceuticals – consultant. Merck, Altor, Amgen, Genentech, Roche, Gerson Lehman Group – speaker bureau. Supergen, Threshold, Biovex, Bristol Myers Squibb, Progen, Ziopharm – research funds). All other authors declare that they have no competing interests.

Keywords

sarcoma; radiation-induced; malignant fibrous histiocytoma

Introduction

External beam radiation therapy (XRT) is an integral and effective modality of treatment for patients with primary breast cancer; however, about 0.16% of treated patients will subsequently be diagnosed with RIS with a median latency of 10 years[1,2]. RIS display a geographic and temporal relationship to prior XRT, representing a small subset (3%) of all soft tissue sarcomas (STSs)[3]. Because of its rarity, treatment recommendations are based on clinical trials evaluating primary sarcoma. However, RIS is much more aggressive and less responsive to treatment than STS. Indeed, the 5-year survival rates for RIS patients are only about 27%, with local recurrence rates as high as 52%[4,5] whereas the 5-year survival rates for primary STS patients are 77%, with local recurrence rates of 19.1%[6]. The prognosis for RIS patients is therefore much worse than that for sporadic STS patients[2]. We have previously reviewed the clinical management of RIS in detail[7].

Malignant fibrous histiocytoma (MFH), now known as pleomorphic undifferentiated sarcoma, is the most common histologic subtype of RIS (36%)[8]. The diagnostic term MFH refers to a variety of pleomorphic sarcoma that shows no definable lineage of differentiation. There is an urgent need for reagents to further our understanding of the highly aggressive nature of RIS in order to better treat and possibly prevent this radiation-induced tumor type. No publicly available cell lines currently exist to facilitate *in vivo* or *in vitro* studies of RIS. The lack of cell lines specific to sarcoma subtypes contributes to a disconnect between the research effort to develop rational therapeutics and the goal of improving patient outcomes[9]. The aim of our study was to establish and characterize a novel MFH RIS cell line suitable for preclinical studies of this rare, aggressive, and often lethal disease.

Methods

Tissue Harvest

The source of the cell line was a 52-year-old woman treated for high grade ductal carcinoma in situ (estrogen receptor (ER) positive) with lumpectomy and 66 Gray of XRT who developed a bulky RIS within the previously irradiated field 5 years later. As the resulting RIS was not responsive to treatment with neoadjuvant doxorubicin and ifosfamide, the patient underwent resection of her RIS with an extended radical mastectomy with widely negative margins.

Preoperatively, we had obtained informed consent from the patient for the use of residual tumor tissue, per our institution's Human Subjects Protection Program approved tumor biorepository protocol (#06-0609-04). At the time of surgery, we harvested fresh RIS tissue and snap-froze a portion in liquid nitrogen. We obtained whole peripheral blood from the patient, which was processed for DNA isolation.

The fresh sarcoma tissue was placed in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotics/antimycotics (AA) (Invitrogen, Carlsbad, CA). Immediately upon receipt of the specimen, the tissue was minced and digested for roughly 16 hours at 37°C with 1X recombinant pure collagenase/hyaluronidase (Stem Cell Technologies, Vancouver, Canada). The tissue digest was then transferred to a 50ml conical tube, and spun down at 800rpm for 1 minute. The fatty supernatant was discarded. The cell pellet was resuspended and plated in fresh DMEM with 10% FBS and 1% AA.

Culture Conditions

We maintained all cell lines in an incubator with 10% humidity and 5% CO₂ at 37° C. The synovial sarcoma cell lines HS-SY-II[10] and SYO-1[11], and human foreskin fibroblasts (HFF) were obtained from the American Type Culture Collection (ATCC) by the Cranmer laboratory, authenticated by STR genotyping and grown in DMEM with FBS and AA. All experiments were performed when cells reached 75% to 85% confluency, and all cultures were passaged when the cell culture reached around 80% confluence.

DNA Preparation and Short Tandem Repeat (STR) Profiling

To isolate genomic DNA, UACC-SARC1 and MDA-MB-231 cells at passage 20 were trypsinized (0.05% trypsin-EDTA; Gibco/Life Technologies, Grand Island NY), washed with phosphate-buffered saline (PBS), and pelleted by centrifugation. DNA was extracted from the cell lines, whole blood and sarcoma tumor with the QIAamp kit (Qiagen, Valencia, CA). UACC-SARC1 and the parental sarcoma tumor were profiled for amelogenin and 15 STR microsatellite loci using the Identifiler polymerase chain reaction (PCR) kit (Applied Biosystems, Foster City, CA) at the University of Arizona Genetics Core per manufacturer's specifications. PCR products were separated by capillary electrophoresis on an ABI 3730 DNA analyzer. Electropherogram analysis and allelic values assignment were performed using Gene Marker software (Soft Genetics, State College, PA). Heterozygosity (i.e., detection of different alleles) expressed as a percentage was calculated based on any difference in the number of peaks within the 15 STR loci (excluding amelogenin). The fractional allele percentage of similarity was inferred by dividing the number of identical alleles by 32 (the total number of alleles studied) and multiplying the result by 100[12].

IHC and ICC

10⁶ cultured UACC-SARC1 cells at passage 19 and mouse xenografts were fixed in 10% neutral buffered formalin for 24 hours. Specimens were then embedded in paraffin via our institution's Tissue Acquisition and Cellular/Molecular Analysis Shared Service. Routine hematoxylin and eosin (H&E) staining was performed on 3 micron thick tissue sections. Cell cultures for ICC were trypsinized and resuspended in 15ml of PBS with 2% FBS, then spun at 1500 rpm for 5 minutes. Cell pellets were then fixed overnight with 6 mL of cold 10% neutral buffered formalin (VWR, Radnor PA). The cell blocks for staining were then processed and paraffin embedded per the manufacturer's instructions using the ThermoShandon CytoBlock kit (Thermo Scientific, Fremont, CA).

IHC and ICC analyses were carried out with prediluted monoclonal (M) or polyclonal (P) antibodies to: vimentin (M) (Ventana Medical Systems, Inc. [VMSI], Tucson, AZ); osteonectin (secreted protein acidic and rich in cysteine [SPARC]) (M); (Abnova, Taipei City, Taiwan); α_1 -antitrypsin (P) (VMSI); α_1 -antichymotrypsin (P) (VMSI); CD68 (M) (VMSI); smooth muscle actin (SMA) (M) (Cell Marque, Rocklin, CA); CD34 (M) (VMSI); epithelial membrane antigen (EMA) (M) (VMSI); S100 (P) (VMSI); desmin (M) (VMSI); pancytokeratin 8, 14, 15, 16, 18, and 19 (P) (gift of the Nagle laboratory, mixture of MAK6 and 10:11); ER (M) (VMSI); PR (M) (VMSI); CD117 (anti-c-KIT) (M) (VMSI); lysozyme (P) (VMSI); epidermal growth factor receptor (EGFR) (M) (Zymed, South San Francisco, CA); p53 (M) (VMSI); and CD99 (M) (VMSI); PTEN (M) (VMSI), RB1 (M) (Epitomics, Burlingame CA) and Ki67 clone MIB-1(M) (Dako, Carpinteria, CA).

All steps of tissue section processing and staining were performed using a Discovery XT Automated Immunostainer (VMSI) and VMSI-validated reagents. They included deparaffinization, cell conditioning (antigen retrieval with a borate- EDTA buffer), staining with primary antibodies; detection and amplification, using a biotinylated-streptavidin-horseradish peroxidase (HRP) and diaminobenzidine (DAB) system; and hematoxylin counterstaining.

Images were acquired with a Paxcam 3 camera with PAX-it Digital Image Analysis and standardized for light intensity.

Human-Specific Lamin A/C Immunohistochemistry

Tissue sections from RIS and xenografts were de-paraffinized and re-hydrated using standard protocols in the Tlsty laboratory. In order to reduce the non-specific endogenous peroxidase background staining, sections were treated with 3% H_2O_2 for 10 minutes at room temperature. Slides were subjected to antigen retrieval by microwaving for 10 minutes in citrate buffer (pH=6.0) then cooled down for 20 minutes at room temperature. The sections were incubated for 60 minutes at room temperature with a primary monoclonal rabbit antibody against lamin A/C (Epitomics) diluted 1:500 in Antibody Dilution Buffer (Genemed, South San Francisco, CA), then for 30 minutes at room temperature with HRP Polymer (Thermo Scientific). After 4 washes, the sections were developed for 5 minutes using DAB as substrate. The sections were counterstained in Mayer's hematoxylin.

Matrigel Invasion Assay

The metastatic potential of the UACC-SARC1, HFF, SYO-1, and HS-SY-II cell lines were evaluated using the BD BioCoat Matrigel Invasion Chamber in duplicate (BD Biosciences, San Jose, CA) with wells prepared per manufacturer specifications. 2.5×10^4 cells each were added to the Matrigel Basement Membrane Matrix inserts and control inserts in duplicate and incubated for 24 hours at 37°C. Non-invading cells were removed and the membrane stained with the Hema 3 Staining System (Fisher HealthCare, Houston, TX). Counting of invading cells was facilitated by photographing the membrane through the microscope. Results were quantified as the percent invasion through the Matrigel relative to the migration through the control membrane. All images were counted by a single investigator (WZ). The data was then analyzed using Prism Graph Pad Version 6 (La Jolla, CA) to plot

bar graphs and compare cell lines using a one-way ANOVA test for non-parametric data to compare the mean percent invasion for each cell line.

Clonogenicity analysis

The UACC-SARC1 was diluted to contain one cell in 150ul of the culture medium (RPMI + 10%FBS) (verified microscopically), and 144 wells of two 96-well adherent plates were filled with 150ul of the medium containing one cell. The cell growth was checked daily and the cell colony formations were recorded on day 12 under the microscope.

A sphere formation assay was performed by culturing UACC-SARC1 in 6-well ultra-low adherent tissue culture plates with MammoCult Medium (Stem Cell Technologies, Vancouver Canada). Tertiary tumorspheres were generated by serial passaging non-adherent cultures as per the Stem Cell Technologies technical bulletin for culture of breast cancer cell lines as tumorspheres.

Cytogenetic Analysis of UACC-SARC1

Karyotype analysis was conducted at the Cytogenetics Laboratory in the Department of Pathology of the University of Arizona. A T-25 flask (Thermo Fisher Scientific, Waltham MA) of UACC-SARC1 cells in complete medium was trypsinized then re-plated onto 8 separate culture dishes in order to provide mitoses for cytogenetic analysis. 48 hours after trypsinization, processed cells were G-banded then analyzed at 1000X magnification under a light microscope.

CGH

An Agilent (Santa Clara, CA) human CGH array with 169,628 features was processed in an R environment for statistical computing and graphics rendering[13]. Raw hybridization signals were normalized using the Bioconductor[14] package snapCGH[15]. Genomic regions with amplifications or deletions were identified using an algorithm implemented in the DNACopy package[16]. *P* values less than 0.01 were considered significant. Positive regions were defined as regions covered by at least 5 probes. One microgram of DNA from RIS tumor, UACC-SARC1, and peripheral blood were compared to reference normal female human DNA. Features were annotated using the Human May 2004 (NCBI Build 35/hg17) assembly.

Sequencing

DNA derived from the UACC-SARC1 cell line, the RIS tumor, peripheral blood, and reference human male DNA were analyzed at 740 known mutation hotspots using the Ion Ampliseq Cancer Panel consisting of 190 amplicons derived from 46 genes (Ion Torrent Systems, Guilford CT). Genomic DNA was amplified, bar-coded, pooled and sequenced on the Ion Torrent Personal Genome Machine using primers and reagents according to manufacturer's protocols. Briefly, 10 nanograms of the same genomic DNA used for aCGH described above were amplified in a multiplexed PCR reaction. The primer-derived DNA was digested from the amplicons and bar-coded using sequencing adaptors ligated to the amplicon ends. The amplicon pools from the four samples were diluted and mixed in equimolar amounts to produce a library. Sequencing templates were generated from the

library by emulsion PCR and template positive beads were selected and loaded onto an Ion Torrent 318 chip for sequencing. Data were analyzed using Ion Torrent Server Suite version 2.0 and Ampliseq Variant Caller plugin v2.0.1.1 with the two normal samples being used to remove false positives.

Severe Combined Immunodeficient (SCID) mouse colony and procedures

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. A SCID mouse colony was developed at the University of Arizona using SCID mice obtained from Taconic (Germantown, New York). UACC-SARC1 cells were trypsinized at roughly 80% confluence, spun down at 800 rpm, and resuspended in 2% FBS saline for counting by hemocytometry and trypsin inactivation. The cells were then re-spun at 800 rpm, and the cells were resuspended in a 1:1 mixture of Matrigel (BD) and sterile saline.

Cells were resuspended in a 1:1 mixture of Matrigel (BD) and sterile saline. 5×10^6 cells or 10^7 cells were injected subcutaneously (SC) on the flank of 8-week-old SCID female mice. The tumors grew for 90 days, then the mice were sacrificed and subjected to gross necropsy. The tumors were harvested for FFPE block preparation. All procedures were completed in accordance with the University of Arizona Institutional Animal Care and Use Committee (IACUC) to the Experimental Mouse Shared Service (EMSS) of the Arizona Cancer Center.

Cell Cycle Analysis

UACC-SARC1 cells grown in a T75 flask (Thermo Fisher Scientific, Waltham MA) were detached with 0.05% trypsin-EDTA. Trypsin was deactivated with DMEM containing serum. The cell suspension was transferred into a 50 ml conical tube that was filled with cold PBS and spun down at 1,500 rpm for 10 minutes. The medium was aspirated and the cells were fixed by adding 1 ml ice-cold 70% ethanol. Fixed cells were spun down, resuspended in 1ml cold PBS, supplemented with 1/20 volume of 10mg/RNase A and 1/40 volume of 1.6mg/ml propidium iodide dissolved in water and incubated at 37°C for 30 minutes. Flow cytometry cell cycle analysis was carried out using a FACScan (BD).

Growth curve

UACC-SARC1 was thawed from a fresh batch taken from liquid nitrogen storage. The youngest passage number was used (passage 21). After thawing, the cells were re-suspended in fresh DMEM+10% FBS+1% AA in a T75 flask and were allowed to grow for 5 days in the incubator at 37°C/5% CO₂/92% humidity to allow adequate time for re-animation. After cells reached 80% confluency, the flask was trypsinized with 2 ml of 0.05% trypsin for 5 minutes. After adequate trypsinization, 6-7 ml of medium was added to stop the reaction. The cells were transferred to a 15ml conical tube and were centrifuged at 1100 RPM for 5 minutes. The pellet was re-suspended in 5ml of medium and cells were counted with a hemocytometer. Cell suspension was diluted with medium to achieve a seeding concentration of ~30,000cells/ml. 1.0ml of our calculated concentration was seeded into 5 T25, 5 T75 and 3 T175 flasks (Thermo Fisher Scientific, Waltham MA) labeled day 2-13 and placed in the incubator. Every 24 hours, 1 flask was counted with the hemocytometer twice. The average was taken to establish the concentration of cells in the flask and was

multiplied by the re-suspension volume to give the total number of cells per flask. These numbers were then plotted to establish the growth curves using Prism GraphPad Version 6.

NOD/SCID gamma mouse model

All NOD/SCID gamma mouse studies were performed according to the protocols approved by the University of Southern California Institutional Animal Care and Use Committee. Experiments were conducted in strict compliance with IACUC regulations. Mice showing any signs of distress or pain were humanely sacrificed. Female NOD/SCID gamma mice were purchased from Jackson Laboratory (Bar Harbor, ME) and were allowed to acclimate for two weeks prior to being used for our studies.

Wild type and GFP/Luciferase tagged UACC-SARC1 cells were cultured in DMEM supplemented with 10% FBS in T75 flasks. Flow cytometry was used on confirm that all UACC-SARC1 cells used for this *in vivo* model were GFP/luciferase positive. When the cells reached 85% confluency, they were trypsinized with 1.5ml 0.05% trypsin, followed by determination of the concentration of cells by hemocytometry. Cells for subcutaneous injection were mixed with Matrigel to obtain a 1:1 cell-Matrigel mixture containing proposed cell number in 100ul of the mixture. Two cellular concentrations were prepared of both the wild type and GFP/ Luciferase tagged cells for mouse modeling, 5×10^5 (low dose) and 5×10^6 (high dose) cells, which were subcutaneously injected into ten 6-week old female NOD/SCID gamma mice. Six mice were injected with the GFP/ Luciferase tagged cells (3 mice with 5×10^5 (low dose) and 3 mice with 5×10^6 (high dose). Additionally, four mice were injected with wild-type untagged cells - 2 mice with 5×10^5 (low dose) and 2 mice with 5×10^6 (high dose) cells. Tumor growth was monitored twice a week with digital calipers for the mice receiving subcutaneous injection.

Ten additional 6-week old NOD/SCID gamma mice were used for an intra-cardiac injection metastasis model as adapted from Gupta *et al*[17]. Flow cytometry was used on confirm that all UACC-SARC1 cells used for this *in vivo* model were GFP/luciferase positive. Wild type and GFP/Luciferase tagged UACC-SARC1 cells were cultured in DMEM supplemented with 10% FBS in T75 flasks. When the cells reached 85% confluency, they were trypsinized with 1.5ml 0.05% Trypsin, followed by determination of the concentration of cells by hemocytometry. Cells were resuspended in PBS and two cellular concentrations of both the wild-type and GFP/Luciferase tagged cells 5×10^4 and 1×10^5 were prepared in a total volume of 50ul for direct injection into the cardiac left ventricular outflow tract of anesthetized mice under ultrasound guidance (Vevo 2100, FUJIFILM, VisualSonics Inc., Toronto, Canada). Bioluminescence imaging was used to monitor the development of metastatic lesions.

Six mice were injected with the GFP/Luciferase tagged cells - 3 mice with 5×10^4 (low dose) and 3 mice with 1×10^5 (high dose). Additionally, four mice were injected with wild-type untagged cells (2 mice with 5×10^4 (low dose) and 2 mice with 1×10^5 (high dose).

In vivo Optical Imaging

All bioluminescence imaging studies were performed at the University of Southern California Molecular Imaging Center using the IVIS SPECTRUM pre-clinical *in vivo*

imaging system and data analyzed using the Living Image 4.2 software (PerkinElmer, Waltham, MA). Briefly, firefly luciferin (PerkinElmer, Waltham, MA) in sterile saline was injected at 50 kg/mg intravenously through the tail vein and the mice imaged 1.5 min post injection. Bioluminescence data for all studies were normalized and 1-minute exposures were used to generate images. Average radiance was determined for each mouse based on regions of interest.

Results

Clinicopathologic Data

Pathologic analysis of the patient's tumor revealed a high-grade sarcoma with angioinvasion. The clinical characterization found no expression of ER, progesterone receptor, HER2, pancytokeratin, or CD117. The Ki67 index was 30% in the clinical pathology report; this finding was also true in subsequent research laboratory IHC and ICC staining of tumor and UACC-SARC1. Given the histomorphologic appearance and the IHC results, the tumor was clinically categorized as sarcoma, not otherwise specified. A residual 4 cm viable tumor remained in the extended radical mastectomy specimen. Margins were widely negative and the patient remains without evidence of disease five years after surgery.

STR Profiling

STR profiling of UACC-SARC1 cells demonstrated that this line is genotypically a match to its parental tumor, with only minor loss of heterozygosity (Figure 1). Comparing the sarcoma tissue to UACC-SARC1, we found heterozygosity of 46.7% and overall allele percent similarity of 78.1%. Using a relaxed definition of percent similarity (i.e. allowing for a difference of a single STR per locus), we found a percent similarity of 84.4%, which met the criteria for positive identification of a cell line (reference 80%) [12].

IHC and ICC

Figure 2 provides a growth curve (A) and photomicrograph (B) of UACC-SARC1. The doubling time of UACC-SARC1 based on hemocytometer counts was 28.3 hours and the cell line demonstrated logarithmic growth kinetics. Four technical replicates of the growth curves were highly consistent, as shown in Figure 2. The doubling time remained consistent after being cultured to 50 passages to date. By light microscopy, the sarcoma tissue and UACC-SARC1 were morphologically similar, showing an admixture of plump spindle-shaped cells as well as pleomorphic cells that appeared morphologically to be more histiocytoid. IHC analysis of the tumor and ICC analysis of UACC-SARC1 revealed shared expression of vimentin, osteonectin (SPARC), and CD68, but tumor-restricted expression of the histiocyte markers α_1 -antitrypsin and α_1 -antichymotrypsin (Figure 3). The pericytes of normal vessels within the tumor were positive for SMA, while the tumor cells were negative, however UACC-SARC1 cells were focally positive for SMA within the cytoplasm. α_1 -antichymotrypsin was 3+ positive in the cytoplasm of the IHC of the tumor. The ICC cultured cells were negative. For α_1 -antitrypsin, the tissue cells were weakly positive in the cytoplasm while the ICC of cultured UACC-SARC1 cells were negative. Both tissue and cell line were negative for CD34, EMA, S100, desmin, pancytokeratin 8, 14, 15, 16, 18, and 19, ER, lysozyme, EGFR, and CD99 (data not shown).

Transwell Invasion Assay

UACC-SARC1 cells displayed invasiveness through Matrigel and 8-micron membrane pores (Figure 4A and B). UACC-SARC1 was more invasive (mean 86%) than SYO-I (mean 45.1%) or HS-SY-II cells (mean 20%) when compared to control wells. As a negative control, normal human fibroblasts were unable to invade the Matrigel membrane. The difference among mean percent invasion for all 3-cell lines taken together was statistically significant ($p=0.02$).

Cytogenetic Analysis of UACC-SARC1 and Cell Cycle Analysis

Karyotype analysis of UACC-SARC1 cells revealed the presence of two abnormal aneuploid composite cell lines in this G-banded study (Figure 5). The first was hyperdiploid ($>2N$), with a modal range of 48 to 57 chromosomes; this was observed in 12/18 (67%) metaphase cells scored. The second was hypertetraploid ($>4N$), with a modal number of >95 chromosomes; this was observed in 6/18 (33%) metaphase cells scored. The hypertetraploid clone appeared to be a doubling of the hyperdiploid clone, and was, most likely, due to cultural artifact. The $>2N$ spindle-cell sarcoma karyotype[18] is as follows:

48-57,XX,add(1)(p36.1) $\times 2$,del(1)(q25),del(2)(p21p23),add(3)(p13),del(3)(q21),-5,del(6)(q21),+7,del(7)(q22q36),+9,+9,add(9)(p24),add(11)(q21),add(13)(p12),add(13)(q34),del(13)(q12q22) $\times 2$,+14,add(15)(p10),add(15)(p12),add(15)(q26),+19,+19,hsr(19)(q13.4) $\times 2$,del(20)(q11.2),-21,+22,add(22)(q13),+1-4mar[cp7]

Extra copies of whole chromosomes 7, 9, 14, 19, and 22, and losses of chromosomes 5 and 21 were noted. Structural anomalies included losses in 1q, 2p, 3q, 6q, 7q, 13q, and 20q. Cell cycle analysis by flow cytometry found that 62.8% of the cells were in G1; 16.2% in G2; and 21% in S phase fraction (SPF).

CGH

Chromosomes of the sarcoma tumor, UACC-SARC1, peripheral blood, and control DNA (normal male versus normal female) were aligned and compared (Figure 6). Although UACC-SARC1 was genetically similar to its tumor of origin, more probes were affected by changes in copy number in the cell line – the majority of these being losses. Quantitative chromosomal losses and gains for the RIS, UACC-SARC1, and peripheral blood are summarized in a Venn diagram (Figure 7). Raw CGH data were deposited in Array Express (accession E-MEXP-3419).

Sequencing

A total of 8.5 million reads were obtained from the Ion Torrent 318 chip. 5.6 million reads remained after removal of polyclonal, primer dimer, and low quality reads by the Ion Torrent Server Suite software. Reads were evenly split between the four bar-coded samples (1.1-1.6 million reads). This provided enough reads for all 190 amplicons to achieve 100-fold coverage in all samples. False positives were identified using the patient's peripheral blood and the human reference DNA. After removal of false positives, and synonymous mutations, no mutations nor indels were found in either the tumor or UACC-SARC1. The

sequenced genes, amplicons, and hotspots, along with the raw sequence data, are available as supplemental data (Supplemental Tables 1 and 2).

SCID Mouse Xenografts

Tumorigenic properties of UACC-SARC1 were assessed *in vivo*. Tumors formed in 3 of 3 SCID mice injected SC with 5×10^6 UACC-SARC1 cells and in 3 of 3 SCID mice injected SC with 10^7 UACC-SARC1 cells. However, none of those 6 mice showed evidence of metastasis, an observation that correlated with the patient's clinical course. While all xenografts successfully established tumors in SCID mice, these tumors peaked in size at days 5-7 (maximal volume of 85.5 mm^3 for the 5×10^6 UACC-SARC1 cells and maximal volume of 58.5 mm^3 for xenografts inoculated with 10^7 UACC-SARC1 cells). All xenografts grossly regressed to pinpoint tumors after a median of 29 days but all demonstrated viable tumor microscopically. The morphology of the xenografts by H&E staining (Figure 8) closely resembled that of the primary tumor's spindle component, but lacked histiocytic infiltration. The xenograft tumors were moderately well vascularized, but due to the amount of extracellular matrix, the vasculature structures were compressed and not abundantly obvious. The presence of cells of human origin in mouse xenografts was confirmed by IHC after staining with a human-specific lamin A/C antibody (Figure 7). Thus, the RIS tumor showed widespread nuclear membrane staining while the murine tissue showed nuclear membrane staining restricted to the SCID mouse xenograft; the adjacent mouse adipose tissue and stroma exhibiting low non-specific staining but no nuclear staining.

Validation IHC and ICC

To validate our next generation sequencing results with the Ion AmpliSeq Cancer panel and to provide further evidence of the origin of this cell line, additional IHC and ICC was performed and is presented in Figure 9. Ki67 was verified to be 30% positive for both cell line and tumor. Mutant p53 was 1% positive for each. 100% of cells expressed PTEN in both tumor and UACC-SARC1. RB1 was negative (0%) for each.

Clonogenicity Assay

90% (130/144) of the wells had cell growth and at least one cell colony present which originated from single cells. Figure 10 presents a representative image of an adherent colony that originated from a single UACC-SARC1 cell. Similarly, tumorspheres could be generated when UACC-SARC1 was cultured in ultra-low adherent plates for suspension culture and could be passaged to tertiary tumorspheres successfully.

NOD/SCID Gamma Mouse Xenografts and Imaging

Nine of nine (100%) orthotopic xenografts produced tumors (mean 829.3 mm^3). One additional mouse received an orthotopic injection but died for unknown reasons 1 week post-injection (low dose cohort). No significant difference was found for tumor size at the conclusion of the experiment (0.07) and all mice were sacrificed either when tumors grew to 1 cm^3 , mice lost 10% body weight or more, or showed signs of systemic illness. A growth curve comparing low dose to high dose orthotopic injections is shown in Figure 11. No

significant difference in mean growth kinetics ($p=0.96$) or final tumor volume ($p=0.08$) was seen between the high versus low dose cohorts, although there was a trend for larger tumors at 6 weeks in the high dose cohort. The mean survival time for both the low dose and high dose orthotopic cohorts were identical at 46 days. Two mice with high dose injections developed visceral metastasis in the orthotopic cohort – 1 with a liver metastasis and 1 with a pleural metastasis. No mice in the low dose cohort developed gross evidence of metastases. Figure 12 presents a representative bioluminescence scan for our orthotopic model. The mouse shown in 12H had developed a bulky pleural metastasis on necropsy. Mice injected with a higher dose of UACC-SARC1 showed an early dose response with higher levels of luminescence based on normalized comparisons of average radiance for imaging time points up to day 26 (example: A-C vs E-G) but were equivalent by day 32 post-injection (example: D vs H).

Of the mice in the intra-cardiac injection xenografts, 8/10 (80%) produced gross metastasis at necropsy, with the most prominent visceral sites of metastasis being liver ($n=7$), lung ($n=1$), and kidney ($n=1$). Based on bioluminescence, 8/10 (80%) mice had evidence of bony metastasis but this could not be verified based on gross tissue morphology or computed tomography angiography to verify gross bony destruction. Tissues were procured for future histologic studies and molecular pathologic analysis in accordance with our IACUC protocol. Mice injected with a higher dose of UACC-SARC1 showed more numerous and more rapid metastasis. The mean survival for the high dose intra-cardiac injection cohort was 33.8 days, versus 42 days for the low dose cohort. Figure 13 demonstrates the GFP/luciferase positive UACC-SARC1 at numerous sites of distant disease and the rapid progression of this metastasis model.

Discussion

Preclinical studies of RIS, for the purpose of testing novel chemotherapeutic agents, have been limited by the lack of a readily available, well-characterized cell line—suitable for *in vitro* experiments and for *in vivo* generation of xenografts[9]. Cowan *et al.* reported the establishment of 6 RIS cell lines in 1990[19]. Currently, none of their 6 cell lines are publicly available. Their MFH cell line (Stsar 5), like ours, was aneuploid (modal number of chromosomes=56) with somewhat variable and unbalanced chromosomal losses and gains[19]. Both UACC-SARC1 and Stsar 5 showed a hyperdiploid and a hypertetraploid component and exhibited long-term continuous culturing.

However, as compared with the cell lines reported by Cowan *et al.*, UACC-SARC1 displayed a much shorter doubling time. Cell lines specific to sarcoma subtypes are critical for preclinical studies of human disease, allowing *in vitro* and *in vivo* animal model testing of therapeutic agents before phase 1 drug testing in clinical trials. Authentic models of sarcoma subtypes play an important role in evaluating novel therapeutic agents for efficacy against STS[20,21] – modeling STS is complicated by the fact that sarcomas are often a combination of mesenchymal cells and histiocytes and their rarity, particularly for RIS. Our data is most consistent with the establishment of a strain of aggressive RIS that consists of various subtypes of sarcoma cells with exclusion of at least some histiocytic populations.

A novel aspect of our report is the authentication of UACC-SARC1 versus the tumor of origin by STR genotyping. Loss of heterozygosity occurred between UACC-SARC1 and its source tumor. Despite the potential for artifacts introduced by cell culture, we successfully authenticated UACC-SARC1, on the basis of STR profiling. A relaxed definition of percent similarity is justified because loss of heterozygosity is present between UACC-SARC1 and its source tumor. In UACC-SARC1, heterozygosity was found to be 46.7% while the expected heterozygosity of Caucasians at these loci is approximately 70% [22]. However, Masters *et al.* reported that heterozygosity in a series of transformed and cancer cell lines ranged from 48.2%-70.3%, therefore the loss of heterozygosity seen in UACC-SARC1 is not unexpected [22]. Genomic differences may also be due to the fact that the portion of tissue available from the sarcoma tumor is adjacent but not identical to the portion of tumor that served as the source of UACC-SARC1.

Overall, we found more genomic losses in UACC-SARC1 than in the tissue of origin. While CGH and karyotyping showed good agreement, some differences were noted, which may be explained by the fact that CGH detects the average signal present within a population of cells, whereas the karyotyping clearly demonstrates marked tumor heterogeneity. The strength of the CGH results is that we demonstrated that most genomic changes present in the original tumor are still present on the derived cell line. Differences between UACC-SARC1 and its parental tissue may be explained by either selection or genomic instability. Thus, UACC-SARC1 is a relevant model for RIS and is clearly more invasive than the primary synovial sarcoma cell lines SYO-1 and HS-SY-II. It would be preferable to compare UACC-SARC1 to other RIS cell lines; unfortunately no other such lines are available to the scientific community. When comparing the RIS cell line UACC-SARC1 to a metastatic MFH cell line that was not a RIS, both show numerous structural abnormalities on cytogenetic analysis [23]. However, UACC-SARC1 showed a relatively higher number of chromosomal losses versus gains.

Pérot *et al.* reported that the *p53* pathway is implicated in primary sarcoma oncogenesis [24]. Mutant *p53* protein is a common IHC finding in primary MFH sarcomas; however, its role in RIS is less clear [25]. We surveyed for *p53* mutations in the tumor of origin and the derived UACC-SARC1 cell line. However, our IHC analysis did not demonstrate evidence of mutant *p53* protein in either of these samples. Moreover, our Ampliseq surveys for 87 distinct *p53* mutations were negative in both the tumor of origin and UACC-SARC1. Validation IHC/ICC showed no evidence of mutant *p53*, *PTEN* or *RB1*, which supported our Next Generation Sequencing results.

In primary STS with complex genomics, *PTEN* and *RB1* deletions have been reported to be prevalent [26]. CGH and karyotyping of UACC-SARC1 revealed allelic loss of regions of chromosome 13 that contain *RB1*. However, Ampliseq analysis confirmed that the remaining *RB1* allele was wild type. Similarly, CGH revealed a loss of regions of chromosome 10 containing *PTEN*; karyotyping and Ampliseq data did not confirm this loss, which also suggests the possibility of *PTEN* haploinsufficiency. The presence of a haploinsufficiency instead of an overt deletion is further suggested by 100% of cells staining positive for *PTEN* by both IHC and ICC. Since *RB1* and *PTEN* are tumor suppressor genes, their potential haploinsufficiency may help explain the robust growth of this cell line [27].

Sequencing of 740 mutation hotspots across 46 oncogenes and tumor suppressor genes using the Ion Ampliseq Cancer panel revealed no mutations or indels in UACC-SARC1 cells nor in the tumor of origin, which rules out the possibility of aberrant PTEN or RB1 protein. Our data suggests that there may exist additional oncogenes or tumor suppressor genes that may explain the biology of RIS that have not yet been identified. Massively parallel sequencing of UACC-SARC1, tumor and somatic tissue may shed light on the genomics of this rare disease.

UACC-SARC1 was found to have an S phase fraction of 21% by flow cytometry, which is abnormally high. Gustafson *et al.* and Huuhtanen *et al.* studied the prognostic significance of elevated flow cytometric SPF in STS and found that it predicted for poor metastasis free survival[28]-[29].

The World Health Organization Classification of Tumors concluded that there is no evidence of a histiocytic origin for MFH[30]. Consequently, it is not surprising that, while our IHC analysis of sarcoma tissue showed positive staining for CD68, α_1 -antitrypsin, and α_1 -antichymotrypsin, in contrast our ICC analysis of UACC-SARC1 was negative for the histiocyte markers α_1 -antitrypsin, and α_1 -antichymotrypsin. While CD68 is a marker for monocytes and macrophages, MFH sarcoma have been reported to express CD68 in up to 71% of cases [31]. This provides further evidence that UACC-SARC1 is a strain representing a subpopulation of the bulk tumor since the primary tumor displayed both CD68 positive and negative cells while UACC-SARC1 is strongly CD68 positive (Figure 3). The primary tumor was positive for the histiocyte markers α_1 -antitrypsin and α_1 -antichymotrypsin while UACC-SARC1 lacks these markers, providing further evidence that it is a strain of the bulk RIS. Similarly, UACC-SARC1 was focally positive for SMA while its parental tumor lacked this marker. Based on our CGH data, the UACC-SARC1 strain appears to have acquired some additional genetic changes subsequent to the initiation of the parental cell line resulting in the demonstrated loss of heterozyosity.

Single UACC-SARC1 cells seeded in tissue culture were sufficient to produce clones in tumorsphere culture, further demonstrating the aggressive behavior and potential tumor initiating features of this cell line[32]. Further studies will be required to determine if UACC-SARC1 cells have the ability to self-renew, differentiate, and produce metastasis from limited numbers of GFP/luciferase tagged cells, as would be required for more definitive proof of stem cell status[33,34]. Recently, Wang *et al* showed that the side population (putative stem cells) from undifferentiated pleomorphic sarcomas displayed activation of the Hedgehog and Notch signaling pathways and that inhibition of these pathways reduced the proportion of side population cells present[35]. We intend to pursue future studies evaluating the inhibition of specific stem cell signaling pathways using UACC-SARC1.

UACC-SARC1 appears morphologically similar to its primary RIS and its derivative xenografts. While UACC-SARC1 could produce tumors in SCID mice, this was much more robustly demonstrated in more immunodeficient mouse models (NOD-SCID IL2R gamma chain knockout mice). Our *in vivo* experiments demonstrated that NOD/SCID gamma mice served as a more robust xenograft model for UACC-SARC1 than did SCID mice, which is

not surprising given that SCID mice are less immunocompromised. This model progressed quite rapidly for both orthotopic and intra-cardiac injections suggesting that UACC-SARC1 would be useful for testing dose-response to pharmacologic agents.

As lamins are nuclear membrane structural proteins critical for cell function, we provided evidence of the human origin of our xenografts by IHC for human specific lamin A/C. Thus, lamin A/C IHC provides evidence that UACC-SARC1 established xenografts in SCID mice. Ki67 staining evidencing 30% cell proliferation also provides specific evidence of the human origin of UACC-SARC1. The cell line seemed to recapitulate the clinical course of the affected patient as the mice produced tumors *in vivo* but they did not metastasize.

Conclusions

In conclusion, UACC-SARC1 provides a novel model for studies of RIS since it represents a subpopulation consisting of both mesenchymal cells and CD68 positive cells lacking other histiocytic markers while gaining focal expression of SMA. To our knowledge, it is the only publicly available RIS cell line. We have characterized this novel cell line in detail, including STR-based authentication. This information will prove invaluable in validating the results of subsequent RIS studies using this cell line. Sarcoma subtypes are clearly heterogeneous in their biology; as a result, multiple RIS cell lines or strains will be required to demonstrate the full spectrum of disease. In light of our detailed characterization and the close similarity of the RIS cell population UACC-SARC1 to the patient's tumor biology, we propose that it can be successfully used for pre-clinical studies of RIS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We acknowledge L. C. Howard, BS of the University of Arizona Cytogenetics Lab for technical contributions. We acknowledge Drs. Mary Knatterud, PhD, Philippe Gascard, PhD, Thea D. Tlsty, PhD, Debu Tripathy, M.D., and Stephen Sener, M.D. for thoughtful discussions and editorial assistance. We acknowledge Dr. Jianxin Zhao, M.D. for performing IHC for lamin A/C.

Financial Support: The Tissue Acquisition and Cellular/Molecular Analysis Shared Service, the Genomics Core and the Experimental Mouse Shared Services of the Arizona Cancer Center are supported by the National Cancer Institute (NCI) grant CA023074; NCI P30ES06694 also supports the Genomics Core. Seed funds were provided by the University of Arizona Department of Surgery and the University of Southern California (USC) Department of Surgery, as well as the Norris Comprehensive Cancer Center (P30CA014089). Southwest Environmental Health Sciences Center grant ES006694 supports Petr Novak as a bioinformatics consultant. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Cancer Institute or the National Institutes of Health. The USC Molecular Imaging Core was supported by S10 OD01809601 and S10 RR02365301 (to Peter S. Conti).

List of Abbreviations

ATCC	American Type Culture Collection
AA	Antibiotics-antimycotics
ABI	Applied Biosystems

aCGH	Array-Comparative Genomic Hybridization
BD	Becton-Dickenson
CGH	Comparative Genomic Hybridization
DAB	Diaminobenzidine
DMEM	Dulbecco's Modified Eagle Medium
EGFR	Epidermal growth factor receptor
EMA	Epithelial membrane antigen
EDTA	Ethylenediaminetetraacetic acid
ER	Estrogen receptor
EMSS	Experimental Mouse Shared Service
XRT	External Beam Radiation
FBS	Fetal bovine serum
FACS	Fluorescence-activated cell sorting
FFPE	Formalin-fixed paraffin-embedded
H & E	Hematoxylin and eosin
HRP	Horseradish peroxidase
HER2	Human epidermal growth factor receptor 2
ICC	Immunocytochemistry
IHC	Immunohistochemistry
IACUC	Institutional Animal Care and Use Committee
MFH	Malignant fibrous histiocytoma
M	Monoclonal
MAK6	Mouse anti-Cytokeratin 6
PTEN	Phosphatase and tensin homologue
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
P	Polyclonal
RIS	Radiation Induced Sarcoma
RB1	Retinoblastoma 1
SPF	S Phase Fraction
SPARC	Secreted protein rich in cysteine
SCID	Severe combined immunodeficiency

STR	Short tandem repeat
SMA	Smooth Muscle Actin
STS	Soft Tissue Sarcoma
SC	Subcutaneous
VMSI	Ventana Medical Systems Incorporated

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Figure 1. UACC-SARC1 and the RIS it originated from are genotypically matched
STR profiling was used to compare UACC-SARC1 to its tumor of origin.

A) Doubling time growth curve for cell culture

B) Phase contrast microscopy image of UACC-SARC1 (20X)

Figure 2. Growth curve and photomicrograph of UACC-SARC1

A) The doubling time of UACC-SARC1 was 28.3 hours in logarithmic growth phase. Four replicate growth curves with error bars are shown. B) A 20X phase contrast photomicrograph of the primary culture of UACC-SARC1 is provided. The cell line was pleomorphic with many spindle shaped cells, all displaying hyperchromatic nuclei that were large relative to the cytoplasm.

Figure 3. Marker expression of UACC-SARC1 and its parental RIS

IHC of a RIS and ICC of the derived UACC-SARC1 cell line revealed shared expression of vimentin, osteonectin (SPARC), and CD68. Vimentin shows that both the reactive stromal endothelium and the tumor cells are positive in the tissue and the cells are positive in the cultured cells. SPARC shows that the tumor cells are positive 3+ in the cytoplasm and are also very positive in the cultured cells. Smooth muscle actin stains the pericytes of normal vessels within the tumor, showing the tumor cells are negative, however in cell culture they are focally positive within the cytoplasm (see arrows for each). CD68 shows positive

macrophages shown as cell processes within the tumor with arrows demonstrating some obviously malignant cells that are negative for CD68. The cultured cells were strongly positive for CD68 cytoplasmic staining and displayed a grossly bizarre morphology consistent with malignant cells rather than macrophages. α_1 -antichymotrypsin was 3+ positive in the cytoplasm of the IHC of the tumor, indicated by arrows. The ICC cultured cells were negative. For α_1 -antitrypsin, the tissue cells were weakly positive in the cytoplasm (see arrows) while the ICC of cultured UACC-SARC1 cells was negative. Expression of histiocyte markers α_1 -antitrypsin and α_1 -antichymotrypsin is restricted to the tumor.

Figure 4. UACC-SARC1 is a highly motile cell line

A Matrigel invasion assay was performed to compare the motility of UACC-SARC1 to that of two synovial sarcoma cell lines, SYO-I and HS-SY-II. The mean percentage of invasion (blue cells invading through Matrigel) for UACC-SARC1, SYO-I and HS-SY-II cell lines were 86%, 45.1% and 20%, respectively. A) The control membranes for each cell line are shown in the left hand column while the membrane with Matrigel is shown in the right hand column for each of the three cell lines. B) A graphical representation of mean percentage invasion, with error bars, is provided for the three cell lines relative to control wells without Matrigel.

Figure 5. UACC-SARC1 exhibits extensive karyotypic abnormalities
Two representative karyograms of UACC-SARC1 are shown.

Figure 6. CGH analysis of matched RIS tumor, UACC-SARC1 and peripheral blood

A composite of chromosomal alignment is shown. Red indicates a gain and green indicates a loss of DNA. Control normal male DNA is used.

Figure 7. Quantitative representation of chromosome alterations in matched RIS tumor, UACC-SARC1 and peripheral blood

A Venn diagram describing the chromosomal losses and gains for matched RIS tumor, UACC-SARC1, and peripheral blood (each normalized to normal female) is shown. The numbers inside the diagram show the losses and gains for each specimen; the numbers in the lower right corner relate to probes showing neither losses nor gains for any specimen type.

Sarcoma
Tissue Sarcoma
Xenograft Sarcoma
Xenograft
H&E

Figure 8. Detection of cells of human origin in mouse UACC-SARC1 xenografts and RIS tumor Representative 20X fields of human-specific lamin A/C staining in the RIS from which UACC-SARC1 originated and a UACC-SARC1 SCID mouse xenograft are shown. Strong nuclear membrane staining is present in the human tumor tissue and the human derived xenograft component of the murine specimen. A 40X view of an H&E stain of the SCID mouse xenograft is shown.

Figure 9. Validation IHC and ICC

Approximately five years after establishment of UACC-SARC1 at the time of the patient's operation, we performed validation IHC of tumor and ICC of cell line based on markers of interest. All were found to show identical percent cellular staining between source tumor and UACC-SARC1, including Ki67 30%, p53 1%, PTEN 100%, and RB1 0%.

Figure 10. Clonogenicity Assay

UACC-SARC1 plated as single cells produced viable colonies in 90% of the wells (130/144). A representative colony is shown.

Figure 11. Xenograft Growth Curve

UACC-SARC1 orthotopic xenografts were established in NOD-SCID gamma mice (n=9/9).

A) No difference in growth curves was seen for high versus low dose injections mean growth. B) No difference in final tumor size was observed at time of mouse sacrifice between high or low dose cohorts.

Figure 12. GFP Luciferase Imaging of Orthotopic Xenografts

Mice injected with a higher dose of UACC-SARC1 showed higher levels of luminescence based on normalized comparisons of average radiance for imaging time points up to day 26 (A-C vs E-G) but were equivalent by day 32 post-injection (D vs H).

Figure 13. GFP Luciferase Imaging of Intra-cardiac Xenografts

Mice injected with a higher dose of UACC-SARC1 showed more numerous and more rapid metastasis. Images A-C were taken from a mouse injected with 100,000 cells into the left ventricular outflow tract of the heart using ultrasound guidance; this mouse survived 3.5 weeks and was moribund when image C was acquired due to a heavy tumor burden, hence decreased tissue perfusion was expected. Images D-F were from a mouse injected similarly with 50,000 cells; this mouse survived 4 weeks.