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Establishment and Molecular Characterisation of Seven Novel Soft Tissue Sarcoma Cell Lines

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Abstract

Background

Soft tissue sarcomas (STS) are a diverse group of malignancies that remain a diagnostic and therapeutic challenge. Relatively few reliable cell lines currently exist. Rapidly-developing technology for genomic profiling with emerging insights into candidate functional (driver) aberrations raises the need for more models for *in vitro* functional validation of molecular targets.

Methods

Primary cell culture was performed on STS tumours utilising a differential attachment approach. Cell lines were characterised by morphology, immunocytochemistry, proliferation assays, Short Tandem Repeat (STR) and Microarray-based Genomic copy number profiling.

Results

Of 47 STS cases of various subtypes, half formed adherent monolayers. Seven formed self-immortalised cell lines, including 3 undifferentiated pleomorphic sarcomas, 2 dedifferentiated liposarcomas (one of which had received radiotherapy), a leiomyosarcoma and a myxofibrosarcoma. Two morphologically-distinct yet genetically-identical variants were established in separate cultures for the latter two tumours. All cell lines demonstrated genomic and phenotypic features that not only confirm their malignant characteristics, but confirm retention of DNA copy number aberrations present in their parent tumours that likely include drivers.

Conclusion

These primary cell lines are much-needed additions to the number of reliable cell lines of STS with complex genomics available for initial functional validation of candidate molecular targets.

Keywords

Sarcoma, primary cell line, in vitro models, soft tissue sarcoma, array CGH, copy number profiles, Short tandem repeat,

Introduction

Soft tissue sarcomas (STS) are a diverse group of malignant tumours that arise in mesenchymal tissues and represent around 1% of adult human malignancies. Comprising over 50 clinico-biologic/molecular subtypes, the majority of STS subtypes remain a significant diagnostic and treatment challenge (Fletcher *et al*, 2013; Italiano *et al*, 2016). Only a small proportion (approximately 20%) have known specific diagnostic markers such a gene mutations or chromosomal translocations and fewer still possess identified molecular therapeutic targets (Fletcher *et al*, 2013; Taylor *et al*, 2011). The remainder are characterised by pervasive chromosomal instability evidenced by multiple seemingly random somatic DNA copy number aberrations. Elucidation of as yet unknown patterns among these complex genomic abnormalities is believed to be the key to accurate diagnosis and identification of molecular therapeutic targets in these heterogenic tumours (Barretina *et al*, 2010; Taylor *et al*, 2011).

Traditionally, the first step of therapeutic target validation for candidate driver genes or proteins utilises *in vitro* disease models (Taylor *et al*, 2011). Recent improvements in the technology for genomic aberration mapping such as high resolution microarray-based comparative genomic hybridisation (array CGH) and next generation sequencing have led to an increased rate of candidate identification and therefore a growing need to establish a wider range of STS cell lines for functional testing (Barretina *et al*, 2010; Taylor *et al*, 2011). In STS however, there is a limited number of *in vitro* disease models (tumour cell lines) available for functional testing and target validation. Data from large-scale cancer cell line studies such as the Cancer Cell Line Encyclopaedia and Sanger Cancer Cell Line Project showed that less than 2% of the commercially available cell lines studied are derived from STS and the majority of these belong to the translocation-driven subgroup (Barretina *et al*, 2012; Forbes *et al*, 2011).

There has been increasing recognition of the limitations of commercial cell lines as a disease model stemming from reports of poor correlation of the response in these cell lines with *in vivo* tumour behaviour (Cree *et al*, 2010; Kamb, 2010). With cellular adaptation to artificial culture conditions, cell lines have been shown to grow more rapidly than parent tumour cells and acquire phenotypic changes that may alter their therapeutic response, such as dependence on growth factors in culture media or adherence to plastic (Kato *et al*, 2008; Pan *et al*, 2009). Furthermore, heterogeneity of

tumour cell clones, which is characteristic of many cancers is lacking in cell lines in which single clones have been selected for and is widely believed to account for the poor correlation of preclinical and clinical data as the cell lines may not reflect resistant tumour cell clones or cancer 'stem' cells that are believed to be responsible for tumour recurrence and late therapeutic failure (Kamb, 2010).

One widely accepted alternative to traditional established (or commercially available) cell lines is the use of cells cultured directly from tumours (primary cell cultures) as *in vitro* disease models. This is however fraught with many problems (Luca *et al*, 2007). Fresh tumour tissue has to be donated by patients and obtained during surgery with the associated ethical and logistic constraints. When available, the behaviour of primary tumour cells in culture is generally unpredictable with a variable rate of successful establishment (Luca *et al*, 2007).

This paper describes our experience of primary tissue culture of soft tissue sarcoma cells and the establishment and molecular characterization of self-immortalised primary cell lines, including morphologic variants derived from seven soft tissue sarcomas.

Materials and Methods

Ethics Statement

National Research Ethics Committee approval was obtained for the collection and use of tumour tissue (Reference number 09/H1313/52). Written informed consent was obtained from all patients prior to the tumour tissue collection, all tissue was stored according to the principles of the Declaration of Helsinki and used in compliance with the Human Tissue Act 2004.

Tumour Samples

Fresh tumour samples were obtained from 47 patients receiving surgical treatment for biopsy-confirmed STS at Sheffield Teaching Hospitals. Preoperative sarcoma diagnoses were confirmed by pathological assessment of the resected tumours which were classified according to WHO diagnostic categories (Fletcher *et al*, 2013) and reported according to Royal College of Pathologists guidelines (Fisher, 2014). Within 30 minutes of resection, tumours were macroscopically sampled by specialised sarcoma pathologists and collected in sterile phosphate-buffered saline (PBS) with additional tumour samples snap-frozen and stored in liquid nitrogen or at -80ºC until DNA extraction. Normal tissue where available typically as part of a wide resection specimen, was obtained from sites macroscopically distant from the tumour and snap-frozen in liquid nitrogen. .

Establishment of Primary Tumour Cell Cultures

Tumour cell cultures were set up under sterile conditions within one hour of surgical resection by simple mechanical tissue dissociation using RPMI 1640 culture medium supplemented with penicillin (100U/ml), Streptomycin (100µg/ml), amphotericin B (5µg/ml), foetal calf serum (20% v/v) and D-glucose (0.4% v/v). Briefly, a small piece (around 10 × 10 × 5mm) of fresh tumour was placed in a sterile petri dish with a few drops pre-warmed (37^{o}C) culture media and minced with a sterile scalpel until very fine. Minced tumour was then suspended in warm media and centrifuged before transfer in fresh warm media to sterile 25mm^2 tissue culture (T_{25}) flasks and 5mm^2 flat sided tubes (slopes) and placed in a 5% CO_2 incubator at 37^{o}C in 95% humidified air. All cultures were inspected daily using a phase-contrast microscope and media changed as required.

A modification of the differential attachment approach described by Nayak and colleagues (Nayak *et al*, 2000) was used and washes were set up when it appeared that some viable cells in early cultures remained unattached to culture flasks. The media containing non-adherent cells was collected and replaced with fresh media, then centrifuged and the pellet re-suspended in fresh pre-warmed media and transferred into a new T_{25} flask for incubation. Adherent cell cultures were maintained by serial passage with gentle trypsinisation at confluence. In case they were undergoing a crisis period, cultures in which the majority of cells appeared senescent were maintained for at leasta further 3 months with daily visual inspection and the media changed as required.

Cultures were given STS laboratory designations based on the chronological order and year in which they were established and passage numbers were indicated using a ‘p’ prefix. For example, STS 03/10 p2 refers to the second passage of cultures derived from the third tumour obtained in 2010. Cultures that were set up as washes were designated with a ‘w’ prefix to the passage number. For example, cells from the second wash of an original T_{25} flask culture setup and currently in their third passage were designated were designated w_2p3 while cells from a wash of an original slope set up at the same passage were designated w_sp3. Cells from original culture setup and washes were maintained separately with all relevant precautions to prevent cross-contamination. In order to make them more recognisable, the nomenclature of cultures that formed stable cell lines was revised to reflect the STS subtype they represent and their city of origin (see below).

Proliferation Assay and Doubling Time

Cells were seeded at a density of 2 x 10^3 in individual wells of a 96-well plate and the MTT ((4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) proliferation assay was performed as previously described (Canovas *et al*, 2008). The ratio of absorbance in the test wells to control wells (containing 100µl DMSO only) was calculated as relative MTT activity and used as a surrogate for the number of viable cells remaining in culture. Average relative MTT activity from four replicate wells was plotted against time and the exponential growth curve plotted and doubling time calculated using GraphPad Prism® software v6.0.

Short tandem Repeat Profiling

Short Tandem Repeat (STR) Profiling to establish unique genomic identities and exclude cross-contamination of cell lines was performed using services provided by the University of Sheffield Core Genomic Facility. Alleles for 10 human loci including *THO1 D21S11 D5S818 D13S317 D7S820 D16S539 CSFIPO AMEL vWA and TPOX* were assessed and compared with large cell line STR profile databases using the standard match threshold of 80% (Capes-Davis *et al*, 2013)

Microarray-based Comparative Genomic Hybridisation (Array CGH)

Genomic DNA extraction and array CGH were performed using the Agilent® 180K platform as previously described (Salawu *et al*, 2012). Control DNA was extracted from normal tissue obtained from the same patient where available. Otherwise, pooled sex-matched genomic DNA (Promega®) was used. Agilent® Genomic Workbench Software v6.0 was used for copy number data analysis and graphical representation on ideograms.

Immunocytochemistry

Cells were prepared for immunocytochemistry by culturing on sterile glass slides for 48 hours prior to fixing with ice cold acetone-methanol (1:1 mixture). Fixed cells were then pre-treated with Triton (0.1% v/v) and H_2O_2 (3%) prior to immunostaining. Primary antibodies for Cytokeratin, Vimentin, Smooth Muscle Actin (SMA) and CD117 (c-kit) were used (see supplementary data Table S1 for details). The Vectastain® system appropriate for each primary antibody was then used according to manufacturers' instructions for secondary antibody staining and detection. Confirmation of staining pattern was performed by light microscopy at appropriate magnification.

Results

Establishment of Primary Tumour Cell Cultures in various STS subtypes

Forty-seven STS cases comprising 16 different subtypes were set up in culture (see Table 1 for details). Failure to establish adherent cultures or early senescence within the first or second passage (p1 – p2) was observed in ten cases. Later senescence occurred in about a third of cases and was generally observed at two points. The first was around the fifth passage (p4 - p7) and the second, after around ten passages (p9 – p12). All cultures that continued beyond 12 passages went on to become stable self-immortalised cell lines that maintained reliably proliferative cultures for over three years with consistent and reliable proliferation rates. In all but one of these cases (a leiomyosarcoma) that formed stable cell lines, the cells adapted to proliferative tissue culture quite rapidly (within days) and no significant crisis period was seen (discussed later). Also of note was that , long term cultures leading to stable cell lines were derived not from the original culture setups but from washes in three seven tumours and in two of these cases, separate washes led to the establishment of morphologically variant cultures of the same tumours, lending support to the differential attachment approach that was used.

Neoadjuvant local or systemic treatment is expected to adversely impact tissue culture outcomes. It was interesting to note, however that both STS cases in which the patient had received neo-adjuvant chemotherapy were able to establish cells that were passaged up to ten times before subsequent senescence and one of four tumours that received neo-adjuvant radiotherapy resulted in a stable cell line (Table 1). Undifferentiated pleomorphic sarcoma (UPS) was the STS subtype that had the most successful outcome with stable cell lines established in three out of seven cases. Of the UPS cultures that did not result in a stable cell lines, two out of the four had received neoadjuvant treatment. Well-differentiated liposarcoma, on the other hand was the STS subtype that had the poorest culture outcome overall with only one of the seven cases able to establish any cells in adherent culture and these subsequently underwent early senescence (Table 1).

In order to minimise the effects of genomic drift and cultural adaptation in future experiments, cultured cells were frozen at -80^{o}C and subsequently in liquid nitrogen every 3 - 4 passages, within the first 15 passages. Table 2 summarises the nomenclature, clinical and cultural phenotypic characteristics of

the seven tumours that established long term primary cell cultures and which are the focus of the rest of this manuscript.

Morphology and Culture Characteristics

The majority of the STS primary cell lines were composed of cells that were morphologically homogenous (Figure 1A and 1B). The exceptions were Shef-*DDLPS* 01, Shef-*UPS* 02 and Shef-*DDLPS* 02 (Table 2). Cells in the former two cultures were mostly spindle-shaped but showed significant variation in their size and shape (Figure 1C). Neither showed distinct colony formation while in adherent culture. Shef-*DDLPS* 02 cultures on the other hand were dimorphic with one clone of long, spindle-shaped cells that were more numerous and a second population of rounded, histiocyte-like cells with distinct nuclei. Both cell populations tended to grow in tight colonies (Figure 1D). In common with Shef-*DDLPS* 02, Shef-*LMS* 01 and Shef-*MFS* 01 cultures were composed of two distinct morphologic cells populations (variants), but these were established as separate morphologically homogenous cultures.

The first Shef-*LMS* 01 clone was established as a wash from an original slope setup following an approximately 8-week period of crisis and designated, w_s variant. These cells had a rounded histiocyte-like morphology in adherent culture and grew in distinct, tight colonies (Figure 1E). A subsequent clone became established independently (after around 12 weeks of crisis) in the first wash of an original T_{25} flask set up and designated the w_1 variant. They were slightly longer, spindle shaped cells that also formed tight colonies (Figure 1F). Similarly, Shef-*MFS* 01 cells established in the initial and subsequent washes derived from the original T_{25} flask set up, designated w_1 and w_2 respectively, showed distinct appearance in culture. While the former comprised cells with a rounded, histiocyte-like morphology that grew in distinct colonies (Figure 1G), the w_2 variant cells had a more polygonal morphology and formed less-distinct colonies (Figure 1H). STR profiling of the morphologic variants in both tumours showed identical profiles that reassuringly, were significantly distinct from those of all the other cell lines in our laboratory and the examined databases (Table 3). Further details of the culture characteristics are shown in supplementary data (Figures S1 to S7).

STR profiling

STR profiling of primary STS cell lines using 10 loci was used to establish cell line identity and exclude cross contamination. Analysis confirmed unique genomic identities for cell lines derived from all seven cases and matched the identity for those cases with separate morphologically variant cultures (Table 3). There was no evidence of significant relatedness with any other known cell lines or intra-laboratory cross-contamination. Alleles for the *AMEL* locus were also concordant with the known gender of the patients from which all cell lines were derived, adding further confidence in the origin of these cell lines.

Profiling was repeated after a further year in culture (30 – 40 passages) in five cases. All retained overall unique profiles when checked against the cell line databases. Minimal evidence of genomic drift with prolonged *in vitro* culture was observed with only single-locus loss of heterozygosity (LOH) noted in three cell lines despite their characteristic genomic instability (Table 3).

Immunocytochemistry

Immunocytochemistry for vimentin was positive in all seven primary STS cell lines, while cytokeratin was consistently negative in keeping with their mesenchymal origin (Table 2). These results were also concordant with the immunophenotype at diagnosis of the corresponding tumour tissue of origin in all cases. The leiomyosarcoma cell line Shef-*LMS* 01 remained strongly positive for smooth muscle actin (SMA) and negative for c-KIT expression even after 61 passages, supporting its smooth muscle lineage of differentiation (not shown). None of the other cell lines stained positive for c-kit or SMA, except Shef-*DDLPS* 02, a de-differentiated liposarcoma that stained weakly positive for SMA (Table 2).

Ploidy

As expected in tumours known to possess complex karyotypes, metaphase chromosome spreads from all the STS cell lines examined showed significant aneuploidy with chromosome counts as high as 200 in some cases (see supplementary data Table S2). The highest chromosome counts were seen in Shef-*LMS* 01 and Shef-*UPS* 02 that had mean chromosome counts of around 120. It is interesting to note that these cell lines also had the shortest doubling times (Table 2). The lowest chromosome counts were seen in Shef-*UPS* 03 with a mean count of 58 chromosomes.

The majority of the cell lines, including those with very homogenous cytomorphologic appearance on microscopy showed significant intratumour, heterogeneity with wide ranges of chromosome counts in

each metaphase spread. The sole exception was Shef-*UPS* 01 showed a fairly consistent near-triploid chromosome count (supplementary data Table S2). Further characterisation using conventional cytogenetics was not performed in favour of molecular cytogenetic profiling as described below.

DNA Copy Number Profiling

Given the characteristic karyotypic complexity of these STS subtypes, our ability to demonstrate similarities in presumably random somatic copy number abnormalities (SCNA) across the genome serves as an important method of establishing how well a cell line represents its parent tumour. Whole genome copy number profile comparisons of paired DNA samples from each parent STS tumour and at least one of the corresponding primary tumour cell lines was therefore carried out using a high-resolution array CGH platform. Control DNA was obtained from normal viscera such as kidney and uterus that were resected as part of surgical excision in Shef-*DDLPS* 01 and Shef-*LMS* 01, respectively. In all other cases, pooled genomic DNA was used as control. Identical control DNA samples were used for all parent tumour and corresponding cell line pairs that were compared.

Analysis confirmed that all seven cell cultures analysed were related to their parent tumours. The greatest similarity across the whole genome was seen with Shef-*DDLPS* 02, Shef-*UPS* 01 and Shef-*UPS* 02 (Figure 2). While this was expected in the former two, where the cultured cell DNA used for comparison was extracted within the first five passages, that from Shef-*UPS* 02 which was performed after over 40 passages was rather interesting (Figure 2). When examined at higher resolution, even the cell lines that showed the greatest dissimilarity across the whole genome retained striking similarities at certain genomic loci with very similar moving average $\log_2$ratio patterns and identical SCNA breakpoints. Examples as shown in Figure 3 include the deletion on the short arm of chromosome 9 seen in Shef-*LMS* 01, the proximal 2q amplicon in Shef-*UPS* 03 and the 5p amplification of Shef-*MFS* 01. Notably, two of these parent tumours were able to establish more than one morphologically-distinct cell type in long term culture, suggesting that and the dissimilarity in the SCNA profile comparisons may be a reflection of the inherent heterogeneity of the parent tumour DNA when compared with the clonal homogeneity of the corresponding cell culture.

Discussion

Commercial cell lines are a widely used *in vitro* disease model for initial functional validation studies of molecular candidate drivers in spite of their recognised limitations. This is because they are a readily available, endlessly replicating source of tumour material from which results obtained are usually reproducible (Cree *et al*, 2010). Many researchers however believe that successful bench to bedside translation of *in vitro* results is well worth the effort of obtaining primary cell cultures while others use results from primary cultures to augment those from the readily available commercial cell lines, instead of replacing them entirely (Cree *et al*, 2010).

In this study, establishment of primary cell cultures was attempted with all fresh STS tissue collected with a success rate of over 70% confluent cultures and more than half the adherent monolayers able to undergo four passages or more (Table 1). This rate is very comparable to previous studies which reported success rates of between 5 and 33% when cultures were attempted from primary solid tumours and slightly higher success rates from tumour metastases (Gazdar *et al*, 1998; McBain *et al*, 1984; Nayak *et al*, 2000) or xenograft-derived cultures (Dangles-Marie *et al*, 2007; Kamiyama *et al*, 2013). Seven tumours (15% of cases) have established stable cell lines whose genomic and phenotypic characteristics were evaluated and compared to those of their corresponding parent tumours in order to confirm their suitability for functional validation studies. Cells were frozen down at intervals at early passages to minimise culture-related genomic drift when cells are used in experiments.

Among the fourteen cases that failed to establish adherent cultures, eight were well-differentiated or myxoid liposarcomas that had high fat and/or myxoid components relative to cell number, which is believed to have reduced the likelihood of adherent culture using the manual mechanical tissue dissociation applied in this study. Simultaneous use of the explant method of culture establishment (Mitra *et al*, 2013) in some of these cases only yielded slow-growing, fibroblast-like cells that failed to reach confluence (data not shown).

Application of the principle of differential attachment as described by Nayak and colleagues (Nayak *et al*, 2000) led to establishment of long term cultures in washes in three cases (Shef-*DDLPS* 01, Shef-*LMS* 01 and Shef-*MFS* 01) when cells adherent in the original culture setups became senescent. In the latter two cases, it also led to the establishment of separate cultures of two morphologically

distinct variants each (Figure 1 and Table 2). Since both cell populations in these cultures were exposed to otherwise identical culture conditions, it is most likely that the variants represent separate clones present within the parent tumour rather than differential adaptation to culture conditions. Similar observations have been made in multiple cases of primary breast carcinoma tissue culture (McBain *et al*, 1984).

STR profiling which is the current recommended standard for cell line identification (American Type Culture Collection Standards Development Organization Workgroup, 2010) not only reliably confirmed identical profiles for the morphologic variants in Shef-*LMS* 01 and Shef-*MFS* 01, but established unique genomic identities for all the primary tumour cell lines reported in this study (Table 3). LOH events were observed when STR profiling was repeated after around 40 subsequent cell passages, which likely represent genomic drift that would be expected in cells undergoing progressive *in vitro* culture (Capes-Davis *et al*, 2013). The rate of genomic drift has been shown to be higher in cells that possess microsatellite instability (Masramon *et al*, 2006), a phenotype that has demonstrated in several studies of soft tissue and bone sarcomas, where it is believed to be due to their overall genomic instability and not necessarily due to the classical defects in mismatch repair as seen in other cancers (Monument *et al*, 2012). While reassuring, the low frequency of LOH events seen in these cell lines after prolonged culture (Table 3) was therefore rather surprising.

All the cell lines remained proliferative in culture for at least three years and had each been passaged at least 60 times. Assessment of their doubling times showed proliferation rates that are comparable to those of well-known sarcoma cell lines such as SK-LMS1 and U2-OS as well as other tumour cell lines (McBain *et al*, 1984). Similarly, clonal-plating efficiency of the primary UPS and LMS subtypes of these cell lines (data not shown) ranged between 25 and 50% comparing favourably with around 30% efficiency reported for the SK-LMS1 cell line in our lab and by other investigators (Kappler *et al*, 2004; Murphy *et al*, 2008). These results reflect the cancer hall marks of increased proliferation and survival among these primary cell lines (Hanahan & Weinberg, 2011).

Characterisation of primary cells cultures for use as models in target validation studies is essential due to the potential for fibroblast overgrowth in early cultures (Mitra *et al*, 2013) and cross contamination by other established cell lines in longer term cultures (Gillet *et al*, 2013). Morphological characterisation may be unreliable because of the potential effects of an artificial *in vitro* microenvironment on tumour cell morphology. In most cancers therefore, detection of diagnostic

biomarker expression by immunochemistry or flow cytometry are commonly used for characterisation. The common biomarkers of tumour cell lineage such as cytokeratin (CK), vimentin, smooth muscle actin (SMA), S100 and CD34, however have variable sensitivity and are not specific for many STS subtypes (Coindre, 2003; Fisher, 2011). Immunochemistry for panels of these markers however remains relevant to diagnostic practice because when interpreted with the appropriate expertise and as an adjunct to histology, they are helpful for the exclusion of benign and non-mesenchymal tumours as well as suggest differentiation lineages in certain STS subtypes. To this end, the immunochemistry results from this study provided support for a mesenchymal lineage for the established cell lines and were concordant with those of their parent tumours, suggesting that they represent those tumours at least to some extent (Table 2).

All the STS subtypes from which cell lines were developed in this study are known to possess genomic instability and complex karyotypes (Fletcher *et al*, 2013; Taylor *et al*, 2011). All seven cases showed highly abnormal chromosome numbers and in some cases, significant changes in chromosome numbers with increased time in culture that reflects inherent genomic instability that is part of the cancer cell phenotype (Hanahan & Weinberg, 2011). Array CGH, a method that allows the mapping of the complex DNA copy number abnormalities across entire genomes and contributed significantly to the recent WHO classification of STS (Fletcher *et al*, 2013) was therefore used for definitive genomic characterisation of the tumour cell lines. Importantly, it also permits the matching of unique and presumably random genomic aberrations that the cell lines and their parent tumours have in common.

Genomic copy number profiles in all seven long-term cell cultures when compared with the corresponding parent tumours showed overall similarity in log ratio patterns (Figure 2). A number of regions with differences in their SCNA pattern were noted. In line with the clonal evolution model of cancer, the genomic instability that is inherent with these tumours combined with their rapid proliferation is expected to result in significant heterogeneity of tumour cell clones that may be under-represented in the cell line (Anderson *et al*, 2011; Greaves & Maley, 2012). This was likely the case in Shef-*LMS* 01 and Shef-*MFS* 01 where the genome profile of the parent tumour was compared with that of one of two confirmed clonal tumour cell variants in culture (Figure 2; supplementary data Figures S3 and S7). Further, prolonged *in vitro* cell culture in addition to the aforementioned factors

is expected to result in the accumulation of genomic copy number or structural karyotypic aberrations, most of which are functionally neutral i.e. 'passengers' (Gillet *et al*, 2013; Greaves & Maley, 2012).

Overall however, the genomic regions with dissimilar aberrations were few when compared to those regions that showed very similar SCNA patterns with near-identical breakpoints. This was seen even after 40 passages in culture in the case of Shef-*UPS* 02 (Figures 2 and 3) and suggests that these cell lines are a suitable *in vitro* disease model as even large-scale studies evaluating the relevance of established cell lines in various cancers have shown that 'driver' genomic aberrations are nearly always retained in well-established commercial cell lines and *vice versa* despite evidence of genomic drift (Barretina *et al*, 2012; Beroukhim *et al*, 2010; Gazdar *et al*, 2010). Moreover, early passages of all the cell lines in this study that presumably bear a closer genomic and phenotypic resemblance to the parent tumour have been banked and can be used for target validation studies.

Among the seven tumours that established long term cultures, three were undifferentiated pleomorphic sarcomas, representing half of the cases that were obtained of this characteristically aggressive STS subtype (Fletcher *et al*, 2013). The other STS subtypes that formed long term cultures were also of a high grade. This suggests that overall, high grade and aggressive clinical course in STS may correlate with amenability to *in vitro* growth, as was observed in primary cultures of breast (Gazdar *et al*, 1998) and colorectal cancer tissue (McBain *et al*, 1984). The three undifferentiated pleomorphic sarcoma cell lines notably demonstrate very different morphology, doubling time and chromosome numbers, which is not unexpected given that this diagnosis is largely one of exclusion (Fletcher *et al*, 2013) and likely includes a number of as yet undefined biologic tumour entities.

Shef-*DDLPS* 02 was derived from a patient who received neo-adjuvant radiotherapy for dedifferentiated liposarcoma with partial response and the cell line probably represents a radio-resistant clone of cells present within that tumour. It is notable that array CGH analysis of this cell line (and its parent tumour) did not show 12q amplification (typically involving the *MDM2* and/or *CDK4* genes) that is frequently seen well- and del-differentiated liposarcomas and was present in Shef-*DDLPS* 01 (Figure 2) and four other liposarcoma cases in this study (Table 1; array CGH data not shown). This is in keeping with data from large scale genomic studies of STS such as the sarcoma genome project showed that up to 10% of DDLPS have neither *CDK4* nor *MDM2* amplification (Barretina *et al*, 2010). Review of the tumour histology confirmed a biphasic appearance with an area

of atypical adipocytic differentiation and other areas of poorly differentiated sarcoma (Supplementary Figure S5) in keeping with the diagnostic criteria for DDLPS. Further, FISH analysis performed on the diagnostic tissue sample for *MDM2* copy number (data not shown) was concordant with the array CGH results. It is therefore likely that this tumour belongs in this category of DDLPS with as yet undetermined specific genomic aberration.

Given their range of subtypes, clinical, genomic and *in vitro* cultural features, the primary cell lines in this study are potentially much-needed additions to the number of cell lines of STS with complex genomics available that could comprise a drug testing panel akin to the NCI-60 panel that includes only more-prevalent cancers (Shoemaker, 2006; Taylor *et al*, 2011). Further, a radio-resistant STS cell line may be utilised for the study of tumour response to radiation either alone or in combination with sensitising agents, as demonstrated was demonstrated with PARP inhibitors in Ewings' sarcoma cell lines. (Garnett *et al*, 2012).

The results of primary tissue culture in this study show the potential for establishment of short- and long term cell cultures from STS tissue. The need for validation studies in short term cultures coupled with their finite nature and limited supply is however a practical limitation to their use by the wider research community. All the long term cell lines in this study demonstrate genomic and phenotypic features that not only confirm their malignant characteristics, but also confirm the retention of the majority of SCNA present in their parent tumours that likely include 'driver' aberrations. When combined with their rapid proliferation and abundance, they therefore represent an excellent model *in vitro* validation of genomic and transcriptomic targets in STS.

Further, the establishment of separate clones that have been genetically confirmed as belonging to the same tumour in two cases will permit the evaluation of differential responses to therapeutic agents. The combination of cryopreservation of early passages of these characterised cell lines as well as the potential to develop prospective short term cultures from other tumours of the same STS subtypes will serve to mitigate some of the concerns that have been raised with existing commercially available cell lines.. For further information on the availability of these cell lines, interested researchers should please contact the corresponding author.

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Titles and Legends to Figures

Figure 1: Representative Phase contrast micrographs of STS primary cell lines **A:** Shef-*UPS* 01 – spindle shaped cells at passage 69 without distinct colony formation in culture. **B:** Shef-*UPS* 03 cultures at passage 35 showing homogenous cultures of long spindle-shaped cells growing in loose colonies. **C:** Shef-*DDLPS* 01 cultures at passage 71 showing pleomorphic cells without distinct colony formation. **D:** Shef-*DDLPS* 02 cultures at passage 22 composed of a combination of spindle-shaped cells (white arrows) and round, histiocyte-like cells (black arrows), both growing in distinct colonies. **E and F**: Cells derived from Shef-*LMS* 01 growing in separate cultures designated Ws (passage 69) and w1 (passage 56), respectively. **G and H**: Morphologically distinct cells derived from Shef-*MFS* 01 growing in separate adherent cultures designated w1 (passage 35) and w2 (passage 31) respectively. Scale bars = 100µm

UPS – Undifferentiated Pleomorphic Sarcoma, DDLPS – Dedifferentiated Liposarcoma, LMS – Leiomyosarcoma, MFS - Myxofibrosarcoma

Figure 2: Genomic Copy Number Profile Comparisons of Seven Soft Tissue Sarcoma (STS) Primary Cell lines (shown on the left) with their parent tumours.

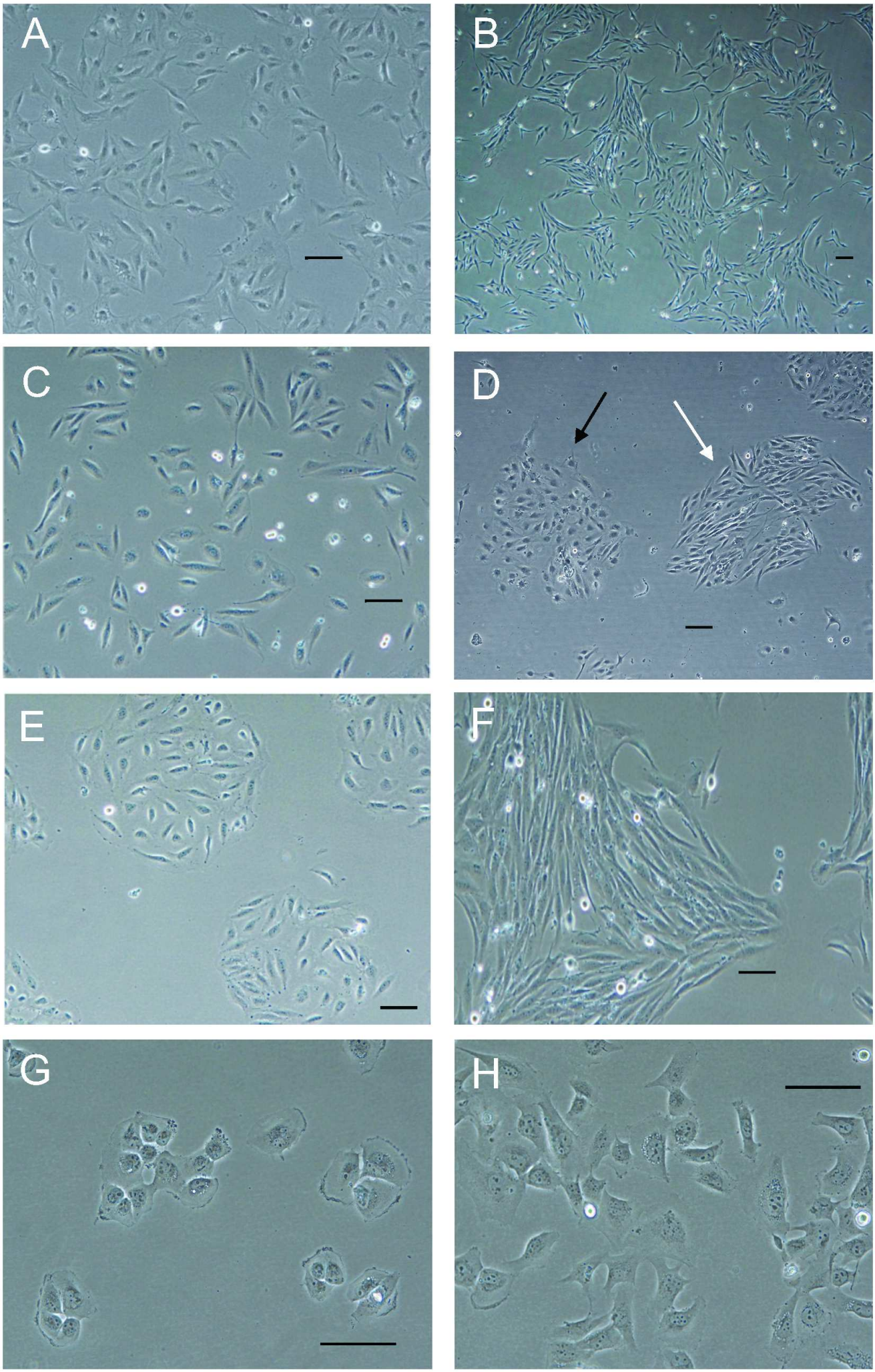
Individual cell lines, STS subtype and passage number at which genomic DNA was extracted are shown to the left of the corresponding autosome ideograms. The overlaid red and blue lines represent the moving average of $\log_2$ ratios of the cultured cells and parent tumour tissue, respectively. Deviations above and below the horizontal baseline represent amplifications and deletions, respectively. Relative amplitude of deviation shows the $\log_2$ ratio and represents DNA copy number at the corresponding genomic locus. Note the close similarity and/or near-identical breakpoints in the moving average patterns in each case over the majority of the genome.

Copy number analysis was performed on the Agilent® 4 x 180K DNA microarray platform and data analysed using Agilent® Genomic Workbench Software v6.0

Figure 3: Selected Chromosome Copy Number Profile Comparisons of Soft Tissue Sarcoma (STS) Primary Cell lines with their parent tumours.

Ideograms of specific chromosomes are as shown at the top of each panel with the corresponding regions at the bottom. The overlaid red and blue lines represent the moving average of $\log_2$ ratios (vs normal genomic DNA) of the cultured cells and parent tumour tissue, respectively. Deviations above and below the horizontal baseline represent amplifications and deletions, respectively. Amplitude of deviation shows the relative $\log_2$ratio and represents relative DNA copy number. Note the close similarity and/or near-identical breakpoints in the moving average patterns in each case.

Copy number analysis was performed on the Agilent® 4 x 180K DNA microarray platform and data analysed using Agilent® Genomic Workbench Software v6.0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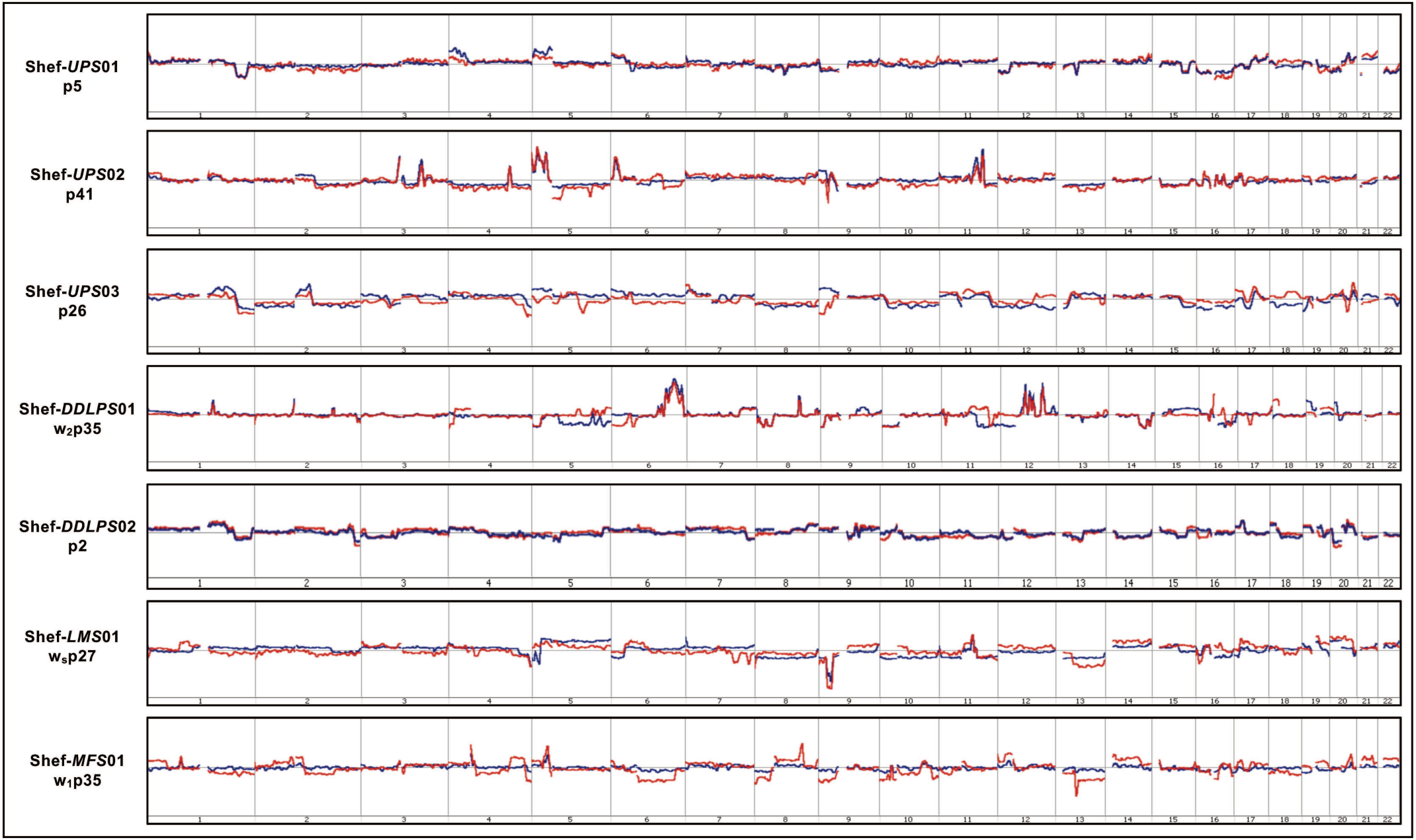
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Shef-*UPS*02 p41
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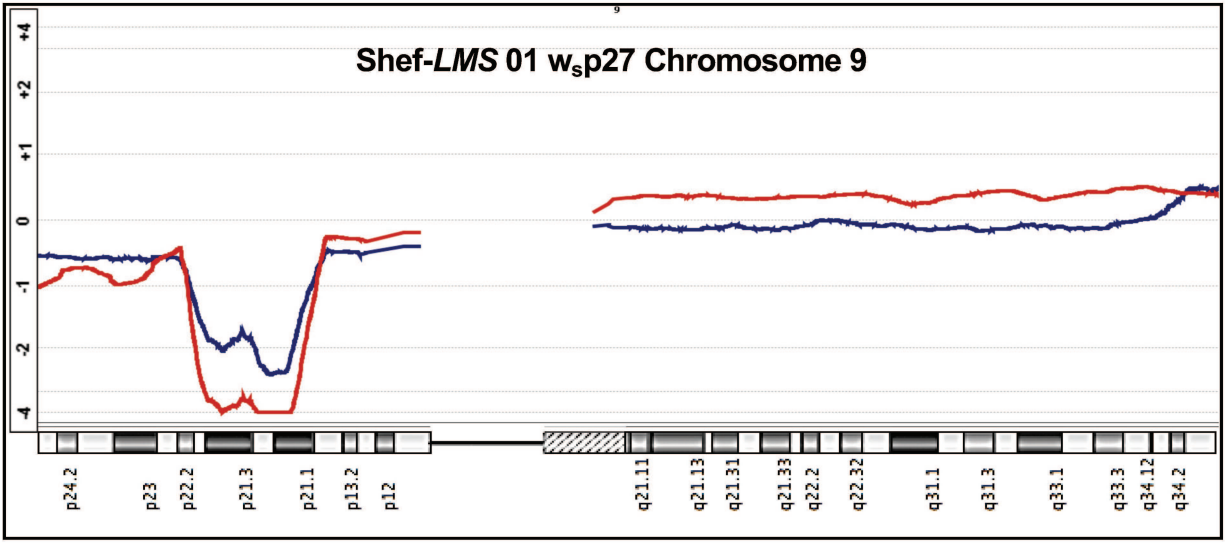
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+2
+1
0
-1
-2
-4
p24.2
p23
p22.2
p21.3
p21.1
p13.2
p12
q21.11
q21.13
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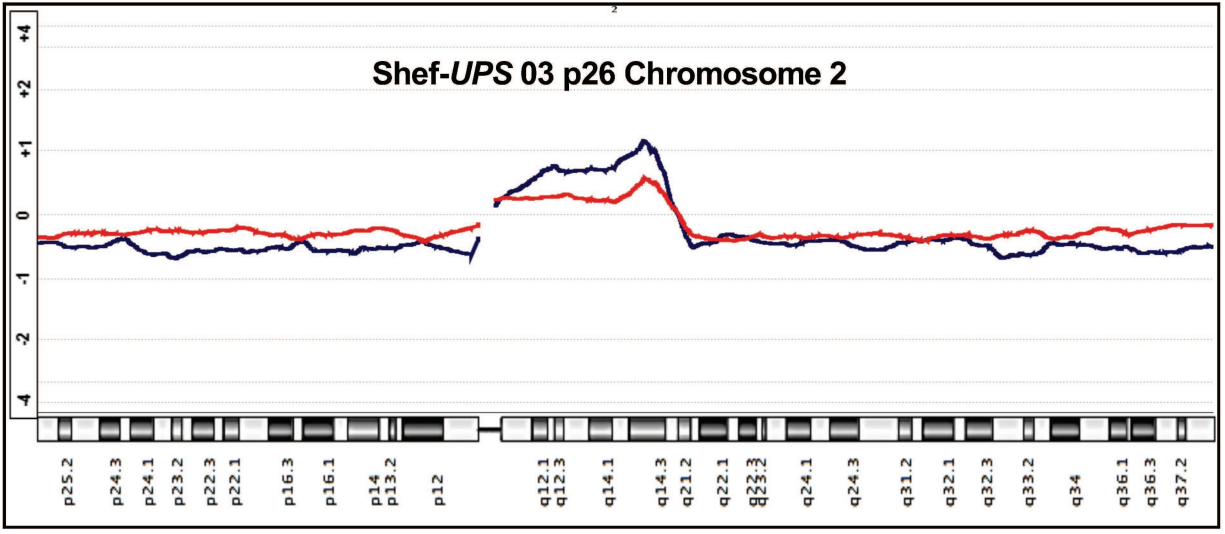
q34.2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+4
+2
+1
0
-1
-2
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p25.2
p24.3
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p22.3
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p16.1
p14
p13.2
p12
q12.1
q12.3
q14.1
q14.3
q21.2
q22.1
q23.2
q23.3
q24.1
q24.3
q31.2
q32.1
q32.3
q33.2
q34
q36.1
q36.3
q37.2

Table 1: A Summary of Fresh Soft Tissue Sarcoma Subtypes obtained and their Primary Cell Culture Outcomes.						
STS Subtype	**Obtained**	**Not Established in Culture**	**Early Senescence**	**Senescent around p5**	**Senescent around p10**	**Long Term Culture**
Number of Cases						
Alveolar Soft Part Sarcoma	2	-	-	1	1	-
Angiosarcoma	6	-	3‡	2	1	-
Dedifferentiated Liposarcoma	7	3	2	-	-	2*
Ewing's Sarcoma	1	-	-	-	1*	-
Extraskeletal Myxoid Chondrosarcoma	1	-	-	1	-	-
Leiomyosarcoma	3	1	-	-	1	1
Low grade Myofibroblastic Sarcoma	1	-	-	1	-	-
Malignant Peripheral Nerve Sheath Tumour	1	-	-	1	-	-
Malignant Solitary Fibrous Tumour	1	-	-	1	-	-
Myxofibrosarcoma	5	-	3	-	1	1
Myxoid Liposarcoma	1	1	-	-	-	-
Pleomorphic Liposarcoma	2	1*	-	1‡	-	-
Pleomorphic Rhabdomyosarcoma	1	-	-	-	1*	-
Synovial Sarcoma	1	-	-	1	-	-
Undifferentiated Pleomorphic Sarcoma	7	2**	1	-	1	3
Well-differentiated Liposarcoma	7	6§	1	-	-	-
Total	**47**	**14**	**10**	**9**	**7**	**7**

* - *One case received neo-adjuvant radiotherapy or chemotherapy*

** - *Both cases received neo-adjuvant radiotherapy or chemotherapy*

§ - *Three cases were recurrent tumours*

‡ - *One case was a metastatic tumour*

Table 2: Characteristics of Primary Soft Tissue Sarcoma Cell Lines and their Parent Tumours

Cell Line	Laboratory Designation	Morphology	Doubling Time* (hrs)	Age/Gender	STS Subtype	Site	Size	TNM Stage	Immunocytochemistry
Shef-*UPS* 01	STS 14/10	Spindle shaped cells with distinct nuclei No pleomorphism No distinct colony formation	p70 = 40.55	53y/F	Undifferentiated pleomorphic sarcoma	Lower Limb	230mm	pT2b Stage III	Vimentin - positive CK, SMA, cKit - negative
Shef-*UPS* 02	STS 06/11	Pleomorphic mostly spindle shaped cells No distinct colony formation	p91 = 35.38	76y/M	Undifferentiated pleomorphic sarcoma	Lower Limb	170mm	pT2b Stage III	Vimentin - positive CK, SMA, cKit - negative
Shef-*UPS* 03	STS 09/11	Long spindle shaped cells No pleomorphism Loose colony formation	p35 = 63.97	66y/F	Undifferentiated pleomorphic sarcoma	Lower Limb	115mm	pT2b Stage III	Vimentin - positive CK, SMA, cKit - negative
Shef-*DDLPS* 01	STS 09/10	Mostly spindle shaped cells Some pleomorphism No distinct colony formation	w_2p35 = 49.5	68y/F	De-differentiated Liposarcoma	Retroperitoneum	300mm	pT2b Stage III	Vimentin - positive CK, SMA, cKit - negative
§Shef-*DDLPS* 02	STS 20/11	2 distinct cell types in same culture A – long spindle shaped cells B – rounded, histiocyte-like cells with distinct nuclei	p23 = 58.22	70y/F	De-differentiated Liposarcoma	Lower Limb	170mm	ypT2b Stage III	Vimentin, SMA - positive CK, cKit - negative
Shef-*LMS* 01	STS 02/11 w_1 w_s	long spindle-shaped cells No pleomorphism Tight colony formation rounded, histiocyte-like cells with distinct nuclei No pleomorphism Tight colony formation	w_1p54 = 27.44 w_sp63 = 44.62	62y/F	Leiomyosarcoma	Pelvis	135mm	pT1b Stage IIA	Vimentin, SMA - positive CK, cKit - negative
Shef-*MFS* 01	STS 21/11 w_1 w_2	w_1 – rounded, histiocyte-like cells No pleomorphism Tight colony formation w_2 – polygonal cells No pleomorphism Loose colony formation	w_1p35 = 59.93 w_2p31 = 56.30	73y/M	Myxofibrosarcoma	Upper Limb	50mm	pT1b Stage IIA	Vimentin - positive CK, SMA, cKit - negative

§ - patient received neoadjuvant radiotherapy

CK = cytokeratin, SMA = Smooth muscle Actin, cKit = CD117

* - passage number at which proliferation assay was performed is indicated

Table 3: Short Tandem Repeat (STR) Profiles of Primary STS Cultures										
Cell Line/Passage	**THO1**	**D21S11**	**D5S818**	**D13S317**	**D7S820**	**D16S539**	**CSFIPO**	**AMEL**	**vWA**	**TPOX**
***Shef-*UPS* 01**										
p31	6,7	27,30	12,13	8,11	8	14	12	X	16	8
p68	6,7	27,30	12,13	8,11	8	13,14	12	X	16	8
***Shef-*UPS* 02**										
p41	6,9.3	29,31	9,13	14	8,11	11	10,11	X, Y	17,18	8
p83	6,9.3	29,31	9,13	14	8,11	11	10,11	X	17,18	8
Shef-*UPS* 03										
P34	9.3	28,31.2	9,12	11	10	9	10	X	17	8
***Shef-*DDLPS* 01**										
w2p35	6	29,32	12,13	8,14	9,10	9,10	10,14	X	17,18	11
w2P70	6	29,32	12,13	8,14	9, 10	9,10	10,14	X	17,18	11
Shef-*DDLPS* 02										
p2	9	29	9	14	10,12	12	12	X	20	9,11
***Shef-*LMS* 01**										
w_1p16	9	27,30	11	14	10,11	11	10,12	X	16	8
w_1p54	9	27,30	11	14	10,11	11	10	X	16	8
w_sp27	9	27,30	11	14	10,11	11	10,12	X	16	8
w_sp63	9	27,30	11	14	10,11	11	10,12	X	16	8
Shef-*MFS* 01										
w_1p35	8	30,31.2	12,13	13	8,9	12	10	X, Y	14,16	11
w_2p31	8	30,31,2	12,13	13	8,9	12	10	X, Y	14,16	11

Profiles comprise the alleles at 10 STR loci. No significant match (>80% relatedness) was found when compared to profiles in the COGcell database (http://strdb.cogcell.org/).

Profiles for morphologically distinct variants derived from two STS cases (shown in bold) are identical

** - STR profiling repeated after one year (30-40 subsequent passages) and disparities in allele matching is highlighted in grey*