A newly characterized human well-differentiated liposarcoma cell line contains amplifications of the 12q12-21 and 10p11-14 regions

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Abstract

While surgery is the usual treatment for localized well-differentiated and dedifferentiated liposarcomas (WDLPS/DDLPS), the therapeutic options for patients with advanced disease are limited. The classical antimitotic treatments are most often inefficient. The establishment of genetically characterized cell lines is therefore crucial for providing *in vitro* models for novel targeted therapies. We have used spectral karyotyping (SKY), fluorescence *in situ* hybridization (FISH) with whole chromosome painting and locus-specific probes and array-comparative genomic hybridization (aCGH) to identify the chromosomal and molecular alterations of a novel cell line established from a recurring sclerosing WDLPS. The karyotype was hypertriploid and showed multiple structural anomalies. All cells retained the presence of a giant marker chromosome that had been previously identified in the primary cell cultures. This giant chromosome contained high-level amplification of chromosomal regions 12q13-21 and lacked the alpha-satellite centromeric sequences associated with WDLPS/DDLPS. The 12q amplicon was large, containing 370 amplified genes. The DNA copy number ranged from 3 to 57. The highest levels of amplification were observed at 12q14.3 for *GNS*, *WIF1* and *HMGA2*. We analyzed the mRNA expression status by real-time reverse transcription polymerase chain reaction for 6 genes from this amplicon: *MDM2*, *HMGA2*, *CDK4*, *TSPAN31*, *WIF1* and *YEATS4*. mRNA overexpression was correlated with genomic amplification. A second amplicon originating from 10p11-14 was also present in the giant marker chromosome. The 10p amplicon contained 62 genes, including oncogenes such as *MLLT10*, previously described in chimeric fusion with *MLL* in leukemias, *NEBL* and *BM11*.

Introduction

Well-differentiated and dedifferentiated liposarcomas (WDLPS/DDLPS) are the most frequent sarcomas in adults [1-3]. Histologically, WDLPS are made up of mature adipocytes, atypical cells and lipoblasts. They are considered as tumors of intermediate malignancy because they demonstrate a high rate of recurrence but rarely metastasize. When mature adipocytes are prominent within a WDLPS, the main differential diagnosis is deep-seated lipoma or intra-muscular lipoma. In approximately 10% of cases, a poorly differentiated component is present and the tumor is classified as DDLPS [3,4]. Most often, the transition between well-differentiated and non-lipogenic areas is abrupt. Difficulties for diagnosing DDLPS arise when the poorly differentiated component predominates. The distinction between DDLPS and poorly differentiated tumors can be hard in such cases.

Molecular cytogenetic analyses are valuable ancillary tools for correct recognition of WDLPS and DDLPS. Indeed, both are characterized by the presence of giant marker or ring chromosomes, containing amplification of the 12q14-15 chromosomal region [5]. The amplified 12q14-15 region consistently contains the *MDM2* gene, located at 12q15, and co-amplification may involve several other genes, including *HMGA2* (12q14.3), *CDK4*, *SAS/TSPAN31* (12q14.1), *YEATS4* [6-8], *CPM* [9] and *FRS2* [10] (12q15).

During the last decade, genomic studies have consistently improved the characterization of WDLPS/DDLPS. However, many points still need to be clarified concerning the biology, pathogenesis and clinical management of these tumors. First, the mechanism of generation of the large marker chromosomes containing the amplified regions remains obscure. Numerous chromosomal regions have been described to be variably co-amplified with the 12q14-15 amplicon [11-13,5,14-21,6,9,8,22]. Moreover, the marker chromosomes present unusual centromere alterations that suggest a peculiar way of formation [15,23]. Second,

while surgery with or without radiotherapy is the usual treatment for localized WDLPS/DDLPS, the therapeutic options for patients with advanced disease are very limited. The classical antimitotic treatments such as doxorubicin or ifosfamide show low response rates [24]. Therefore, there is an urgent need for novel targeted therapies against WDLPS/DDLPS. Several strategies such as those targeting P53 or the JUN/JUNK pathways are currently studied [25,20]. However the lack of model systems impairs the development of new treatments. Indeed, WDLPS/DDLPS are difficult to grow *in vitro*. Most primary cultures do not provide enough material for extensive genomic and pharmacological studies. To our knowledge, only a few human cell lines, such as LS141, DDLS [26] FU-DDLS-1 [27], LS14 [28], LPS695, LP6 [29] GOT3 [30], 93449 and 94778 [15,6], have been described as arising from WDLPS/DDLPS. We report here the molecular and cytogenetic characterization of a novel WDLPS cell line designated 95T1000.

Materials and methods

Clinical information and histological analysis

A 43-year-old man without significant medical history presented in 1990 with a swollen thigh. The tumor was located within the adductor muscles. Histologically, the surgical specimen showed two components: a sclerosing WDLPS zone without any necrotic area or mitotic activity, and a cellular dedifferentiated area containing short spindle cells organized in a cartwheel pattern. Tumor cells of the dedifferentiated area presented hyperchromatic irregular nuclei, with low mitotic activity. Zones of tumor necrosis were present. Transition between WDLPS and poorly differentiated zones was abrupt. The diagnosis was sclerosing WDLPS with dedifferentiated zones of grade II malignancy. The metastatic workup, including a chest computed tomography scan, was negative. Surgery was considered as R0 [31] and adjuvant radiotherapy (50Gy) was delivered.

A local recurrence presented in October 1995. The tumor measured 14 cm x 7.5 cm and was macroscopically well-delineated. Histologically, the tumor presented the features of a sclerosing WDLPS without any necrotic area or mitotic activity (Fig. 1A). Preoperative radiotherapy (45Gy) was delivered. Immunohistochemistry showed strong expression of MDM2 protein (Fig. 1B). A second grade I sclerosing WDLPS recurrence presented in October 1997. The diagnosis of sclerosing WDLPS was established according to the World Health Organization Classification of Tumors [1,32].

Establishment of the cell line and molecular cytogenetic analysis

Fresh tumor material from the first recurrence in 1995 was reserved for cell culture and cytogenetic analysis following surgical excision. Tumor tissue was disaggregated and cells from short-term cultures (10 days) were used for metaphase chromosome preparations according to established procedures [33]. Meanwhile, tumor cells were maintained in culture in RPMI 1640 medium (Gibco BRL, Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal calf serum (Gibco). Karyotype analyses of the 95T1000 cells were performed at passages 4, 10, 12, 31, 34, 54, 59, 60, 61, 69 and 80 (P80). Metaphase cells were analyzed by reverse banding using heat and Giemsa (RHG).

Spectral karyotyping (SKY) was performed on metaphases from P80 according to the manufacturer's instructions (Applied Spectral Imaging, Carlsbad, CA, USA) and as previously published [34,35]. Fluorescence *in situ* hybridization (FISH) analyses were performed on metaphase and interphase cells. The FISH probes used

for our study are listed in Table 1. Bacterial artificial chromosomes (BAC) clones from the Roswell Park Cancer Institute library were selected according to their location in the University of California Santa Cruz genome browser (<http://genome.ucsc.edu/>) and obtained from the Children's Hospital Oakland Research Institute (CHORI, <http://bacpac.chori.org/>). Microscopic analysis was performed using a DM6000B microscope (Leica Microsystems, Wetzlar, Germany). FISH images were processed using the ISIS software (MetaSystems, Altussheim, Germany). Whole chromosome painting (WCP) probes and pan-alpha satellite probes were used according to supplier's recommendations (MetaSystems, Altussheim, Germany; QBIogene, Illkirch, France, respectively). Preliminary FISH data using 12q probes have been published in Italiano et al. (case #16) [6].

Array-Comparative Genomic Hybridization (aCGH) analyses

DNA was extracted using Qiagen Blood and Tissue DNeasy Kit (Doncaster, Victoria, Australia) following the manufacturer's instructions. aCGH analysis was performed on 95T1000 and normal female reference DNA (Promega, Madison, WI, USA) using Affymetrix 250K *NspI* SNP microarrays (nominal average resolution: 1-5 kb), according to manufacturer's instructions (Affymetrix, Santa Clara, CA, USA). Copy number analysis of the 250K *Nsp* data was initially processed using Affymetrix Genotyping Console (GTC3.0.1), and subsequently analysed using the Partek suite of software (St Louis, MO, USA) as previously described [36]. Copy number information was derived after normalizing to the control reference DNA, and chromosome gains and losses were expressed as estimated-relative-copy-number (ECN). The Partek segmentation algorithm used a minimum of 1 feature per region, a P-value threshold of $P < 0.00001$, a signal to noise ratio of 0.3, and an expected range of 0.3 (Table S1).

Real time quantitative RT-PCR (QRT-PCR)

Real time quantitative RT-PCR (QRT-PCR) was used to determine the expression levels of *GLI1*, *DDIT3*, *CDK4*, *TSPAN31*, *MDM2*, *YEATS4*, *WIF1*, *HMGA2*, *CDKN2A* (*p16INK4a* and *p14ARF*), *CCND1* and *RB1*. Total RNA was extracted from the 95T1000 cells (P57) and from short term cultures of normal adipose tissue using TrizolTM (Invitrogen, Carlsbad, California) according to the manufacturer's protocol. The RNA samples were treated with DNA-freeTM (Applied Biosystems, Foster City, California). The quality of RNA was evaluated using the 2100 BioAnalyser and RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, California). One µg of total RNA was reverse-transcribed into cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Each Q-PCR experiment was performed twice using either fluorescein amidite (FAM) dye for *MDM2*, *CDK4*, *DDIT3*, *YEATS4*, *HMGA2* (exons 1-2), *HMGA2* (exons 3-4), *HMGA2* (exons 4-5), *TSPAN31*, *GLI1*, *WIF1*, *p14ARF*, *CCND1*, *RB1* or SYBRGreen dye for *p16INK4a* according to the manufacturer's protocols (ABI PRISM 7500 Detection System, Applied Biosystems). *RPLP0* was used as endogenous control for normalization [37]. QRT-PCR was performed using the TaqMan gene expression assays (Applied Biosystems) (Table S2) as described in Italiano et al. [6]. The primers used for SYBRGreen experiments are described in supplementary Table S3. The comparative threshold (Ct) cycle method was used to achieve relative quantification of gene expression [6].

Results

Conventional and spectral karyotype: hypertriploid genome containing giant marker chromosomes

Cytogenetic analysis of the primary culture of the 95T1000 WDLPS showed a near-diploid karyotype. The number of chromosomes ranged from 45 to 49 after 10 days of culture. One or two giant marker chromosomes were observed in metaphase cells (Fig. 2A). Several structural abnormalities that were not identifiable by conventional banding techniques were also observed. At P80 the karyotype was hypertriploid; the number of chromosomes ranged from 62 to 89, with a modal number of 79. We observed one or two giant marker chromosomes apparently similar to those observed in the primary culture. In addition, several structural and numerical abnormalities were observed (Fig. 2B). These anomalies were too complex to be correctly interpreted by means of conventional cytogenetics and were characterized by SKY analysis that showed a variety of clonal and non-clonal aberrations (Fig. 2C). Both intra- and inter-chromosomal anomalies were observed. The extremities of the giant markers had variable origins: they were most frequently composed of sequences from chromosome 14 and intermittently from chromosomes 1, 5, 6 and 11 (Fig. 2D and Table S4). The SKY method was not appropriate to identify the central region of the giant markers because the dyes were too closely intermixed for providing an interpretable pattern. The co-amplification of sequences from chromosomes 10 and 12 that constitutes the core of these giant markers was eventually identified by aCGH and complementary FISH analyses (see below).

Among abnormally sized chromosomes apparently composed of unique chromosome material, aberrant chromosomes 1, 2, 3, 4, 7, 10, 11, 12, 13, 16, 18, 20, 21 and 22 were frequently observed. In addition to the marker chromosomes with 10p-12q amplification mentioned above, fifteen recurrent derivative chromosomes showing inter-chromosomal rearrangements were identified (Table 2 and Fig. S1). The derivative chromosomes A, F, H, J and O were sometimes duplicated. Most derivative chromosomes were made up of two to three different chromosomes while the derivative B and C resulted from more complex rearrangements.

Evidence for chromosomal instability was suggested by the heterogeneous presence of various derivative chromosomes within the different metaphase cells analyzed (Table 2), as well as variation in the number of giant markers (Table S4).

Amplification of 10p and 12q detected by array-Comparative Genomic Hybridization

The detailed aCGH profile of losses, gains and amplifications was identified across the genome (Fig. 3A and Supplementary Table S1). The most significant imbalances were represented by high-level amplification of chromosomes 10 and 12 (ECN higher than 10). The 10p amplified regions spanned approximately 27 Mb from 10p14 to 10p11.21 and were composed of four distinct amplicons: a large one of 5.4 Mb from 10p14 to 10p12.2, two small ones (10p12.1/ 0.8 Mb; 10p11.22/ 1 Mb) and an isolated focal amplification (31 ECN) of 0.2 Mb at 10p11.21 (Fig. 3B). A total of forty-five segments showing various levels of gain and amplification (from 6 to 55 ECN) and sizes (from 30 kb to 0.7 Mb; average: 0.5 Mb) were identified (Table 3). Overall, these segments included 62 amplified genes.

The amplified regions from the long arm of chromosome 12 spanned 82 Mb from 12q12 to 12q24.21 (Fig. 3C) and consisted of 67 independent amplified segments the size of which ranged from 70 kb to 2.4 Mb (average: 0.4 Mb; total size: 27.8 Mb). The ECN ranged from 3 to 57 (average: 18) (Table 3). Overall, these

segments contained 370 amplified genes including *CDK4*, *TSPAN31* (12q14.1), *GNS*, *WIF1*, *HMGA2* (12q14.3), *MDM2*, *CPM*, *YEATS4* and *FRS2* (12q15). In contrast, *GLI1* and *DDIT3* (12q13.3) were not amplified (Fig. 3C).

No losses were observed except for a 1.3 Mb segment at 20p13 and a 3.4 Mb segment at 20q13.32-q13.33. On the proximal side of the 20q13 deleted segment, a moderate gain (4 ECN) of 16 Mb was observed. Chromosomes 4, 13 and 17 showed similar profiles characterized by moderate gain of long segments (from 3 to 7 ECN) across the full chromosome length (Table 3, S1).

Comprehensive structural analysis of amplified 10p and 12q segments within the giant marker chromosomes

The 10p and 12q amplified sequences were co-located and intermixed in the central region of the giant marker chromosome as well as in a few other marker chromosomes (B, C, G, J and O) as shown by FISH analysis with WCP 10 and 12 probes as well as several locus-specific probes, such as RP11-150J3 (*FRMD4A*; 10p13), RP11-177H22 (*MLLT10*; 10p12.31), RP11-269B1 (*NEBL*; 10p12.31) and *MDM2* (12q15) (Fig. 4). Discontinuities in the 10p and 12q amplified regions were detected by shifts of amplification levels nearby or within several genes: for instance, BAC clone RP11-34I5 (10p12.31) that contained the 3' region of *NEBL* did not show an amplified signal in contrast to the high amplification detected for clone RP11-269B1 that contained most part of the *NEBL* gene. A structural rearrangement was also detected in the vicinity of *HMGA2*: while probes containing exons 1 to 5 (RP11-299L9, RP11-23C9 and CTC-782I9) and the telomeric side of the 3' extremity of *HMGA2* (RP11-118B13) showed highly amplified signals, only a few gained copies were observed with RP11-30I11 probe, located on the centromeric side of the 5' extremity of *HMGA2* (Fig. 4F). In addition, it is noteworthy that the giant marker did not contain any alpha-satellite sequences, suggesting the presence of an analphoid neocentromere (not shown).

Secondary chromosomal anomalies: complementary FISH analyses

The centromeric probes for chromosomes 3, 7, 9 and 17 showed a signal pattern consistent with the hypertriploid karyotype (Table 1). Four copies of each were identified on normal and abnormal chromosomes, respectively. The probes for chromosome 9 showed not only a pattern consistent with hypertriploidy, but also the presence of structural rearrangements. The *CDKN2A* locus (9p21) was disomic, indicating a relative loss of the 9p21 region since four chromosome 9 centromeres were observed. This loss was not detected by aCGH analysis, presumably because of a low statistical significance value related with the polyploidy of the specimen (Table S1). *MAP3K5* (*ASK1*) (6q23.3) and *JUN* (1p32.1) loci showed no imbalance, in concordance with the aCGH results (Table 1, S1). The *CCND1* gene was duplicated on abnormal derivative chromosomes 11. The interstitial gains of chromosome 13 long arm detected by aCGH was confirmed by FISH analysis, which showed 2 to 6 signals per cell for the CTD-2328A22 probe (13q34) while more than 10 signals were observed with the RP11-313L9 probe (13q33.3-13q34) (not shown).

Real time quantitative RT-PCR (qRT-PCR)

We have detected strong overexpression of the following genes: *MDM2*, *CDK4*, *YEATS4*, *HMGA2*, *TSPAN31* and *WIF1* and no overexpression of *GLI1* and *DDIT3* (Table 4). Among the overexpressed genes from 12q region, *WIF1* showed the highest fold change (1733, in comparison to normal adipose tissue). All the five exons of *HMGA2* showed overexpression (fold change values: 317 for exons 1 and 2, 65 for exons 3 to 5, respectively).

Genes of the RB1 pathway, such as *CCND1*, *CDK4*, *CDKN2A* (*p14ARF* and *p16INK4a*), were overexpressed whereas *RB1* was not.

Discussion

We describe a novel WDLPS cell line designated 95T1000. This cell line was established spontaneously from primary cultures of a recurring sclerosing WDLPS and contained high-level amplification of the 12q14-15 region located on supernumerary giant marker chromosomes. The patient had received adjuvant radiotherapy following the excision of the primary liposarcoma, and also a preoperative dose of 45 Gy prior to the excision of the first recurrent tumor that was used for establishing the cell line. This first recurrence did not present the features of a radiation-induced liposarcoma, since it was histologically similar to the primary tumor and with a near diploid karyotype, including the characteristic giant markers containing 12q14-15 amplification. It is possible that radiation contributed to the occurrence of several clonal and non-clonal chromosomal alterations seen in addition to the large markers. The giant marker chromosomes were retained along the subcultures and were still present after more than 80 passages.

The 12q14-15 amplification is fully characteristic of WDLPS/DDLPS. It constitutes the genetic hallmark of this group of tumors [13,38,39,14,5,15,16,40,21,26,9,41,29,30,22,10]. Consequently, detection of 12q amplified genes is widely used for distinguishing WDLPS/DDLPS from other tumors. This detection can be done by FISH [15], Q-PCR [42] or aCGH [4] and indirectly by immunohistochemistry [43,44]. *MDM2* has early been identified as the main driver gene of the WDLPS/DDLPS amplicon since it is consistently amplified in all cases of WDLPS and DDLPS [43,10,45,46]. The overexpression of *MDM2* protein generated by the genomic amplification inactivates TP53. Indeed, *MDM2* targets TP53 degradation toward the proteasome, and inhibits P53-mediated transactivation [47]. Pharmaceutical compounds, such as nutlin, are currently being tested for the purpose of blocking the P53 inactivation mediated by *MDM2* [48].

Despite its obvious major contribution, the amplification of *MDM2* does not seem to be sufficient by itself to account for the pathogenesis of WDLPS/DDLPS. Other genes from the 12q14-15 region are co-amplified and co-expressed with *MDM2* [7,49-51,10]. Similarly, a number of previously described WDLPS/DDLPS cases, have identified giant marker chromosomes of comparable size to that in the 95T1000 cell line that also contained amplification of *CDK4* and *HMGA2*. *CDK4* is not consistently amplified in WDLPS/DDLPS. It is amplified only in approximately 85% of cases [6,7,1,30,10]. The absence of *CDK4* amplification in WDLPS has been correlated to low-grade lipoma-like tumors of peripheral location and a better outcome [7]. In the present case although the primary tumor was located peripherally, it had a high potential of recurrence that might be related to *CDK4* amplification and overexpression.

We observed amplification of the 5 exons as well as the 3' region of *HMGA2* and this amplification resulted in overexpression of the whole gene. It is noteworthy that the region immediately proximal to the 5' part of *HMGA2* was not amplified (Fig. 4F). Structural rearrangements of *HMGA2* have initially been described in lipomas where they often consist in breakage within the third intron followed by fusion with various partner genes [52-54]. It was later shown that anomalies of *HMGA2* were not confined to benign adipose tumors since *HMGA2* was recurrently amplified, rearranged and overexpressed in WDLPS/DDLPS [55-57,6,58,59]. Although various ectopic sequences can be fused to *HMGA2* in WDLPS [56], it does not appear that *bona fide* fusion genes are present in WDLPS as they often are in lipomas. However it has been shown that in lipomas as well as in other tumors overexpression of *HMGA2* is not always generated by fusion genes [60-63].

Overexpression of *HMGA2* is a feature shared by both lipomas and WDLPS, though resulting from distinct events. The exact underlying mechanisms of dysregulation that makes *HMGA2* a central gene in differentiated adipose tumors still remain to be elucidated.

Overall, the size of the 95T1000 12q amplicon was large, comprising 370 genes. This is more than twice the number of amplified 12q genes found in the WDLPS cell line described by Persson et al [30], but similar to the number observed in 93T449 and 94T778 WDLPS cell lines [23] [and D.M.Thomas, personal communication]. Among these amplified genes, we have found amplification of *TSPAN31*, *GNS*, *WIF1*, *CPM*, *YEATS4* and *FRS2* as recurrently described in WDLPS/DDLPS [30,6,7,10].

In addition to the amplification of 12q genes, we have found a high level amplification of the 10p chromosomal region. The finding of co-amplification of at least one chromosomal region other than 12q14-15 [12,13,5,14,16-21,6,9,8,22] is a recurrent feature of WDLPS/DDLPS. These “extra-12q14-15” co-amplified regions are variable among patients. The most frequent and most studied are 6q23, 1p32 and 1q23-24. While the amplification of *MAP3K5* at 6q23 and *JUN* at 1p32 has been correlated to the presence of a poorly differentiated component [40,17,41,29,22], the significance and role of other genomic regions that are co-amplified with 12q14-15 is not elucidated. We have emitted the hypothesis these co-amplified regions might provide neocentromeric sequences to the giant markers that lack alpha-satellite sequences [15,23]. The 1q23-24 amplicon [30,56] has been recently suggested to be associated more frequently with WDLPS/DDLPS lacking *CDK4* amplification [64,7]. The 10p11-14 region is rarely co-amplified with 12q in WDLPS/DDLPS, only a handful of cases have been described [65,22]. In the 95T1000 cells, the 10p amplicon contained 62 genes. It was smaller than the 12q amplicon (27 Mb) but reached similar ECN. It also displayed a discontinuous pattern. Among the amplified genes, several of them are of interest because of their oncogenic functions. In particular, we found amplification of the oncogene *MLLT10*. This gene, previously called *AF10* encodes a transcription factor and is a fusion partner of *MLL* in acute leukemias with t(10;11)(p12;q23) [66,67]. It has also been described to fuse with *PICALM* (11q14.2) in various hematologic malignancies, including acute lymphoblastic and myeloblastic leukemias and diffuse histiocytic lymphomas. In addition, *MLLT10* is a susceptibility locus for meningioma [68]. The *NEBL* gene that codes for nebulin protein highly expressed in cardiac muscle [69] is also amplified in 95T1000 cells. This gene is located closely nearby *MLLT10* and has also been described to be a fusion partner of *MLL* in a case of infant leukemia [70].

Another interesting oncogene contained in the 10p amplicon was *BMII*. It encodes a polycomb-group protein involved in a multiprotein complex that represses the transcription of developmental genes, such as *Hox* genes. *BMII* is required for proliferation of both normal and leukemic stem cells [71]. It is expressed in a variety of malignancies such as lymphoma, leukemia, carcinoma, medulloblastoma as well as glioblastoma and has been shown to be moderately amplified in some cases of mantle cell lymphoma and gained in gliomas [72].

In conclusion, we describe here the genetic features of a novel WDLPS cell line characterized by typical 12q amplification. We provide a comprehensive description of the chromosomal anomalies present in these cells by using a panel of molecular cytogenetics methods. Notably, this cell line harbors an original amplification of the 10p11-14 region. Future studies on genes contained within this region will hopefully shed light on the pathogenesis of WDLPS.

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Conflict of interest statement

We declare that we have no conflict of interest.

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Legends

Figure 1. Histological features and anti-MDM2 immunohistochemical staining of the 95T1000 well-differentiated liposarcoma. **(A)** Histological features of well-differentiated liposarcoma of the sclerosing subtype, characterized by adipocytic cells admixed with thick fibrous septa containing spindle cells with nuclear atypia (Haematoxylin-Eosin, magnification x100). **(B)** Strong nuclear immunohistochemical staining of spindle cells with anti-MDM2 antibody, magnification x200).

Figure 2. Chromosomal analysis. **(A)** Representative RHG-banded near-diploid metaphase cell of the primary culture containing 47 chromosomes. The arrow indicates the supernumerary giant marker. **(B)** Representative RHG-banded metaphase cell at passage 80 (P80) containing 83 chromosomes including the giant marker chromosome (arrow). **(C)** False-color Spectral karyotype (SKY) of a hypertriploid metaphase cell at P80. Complex numerical and structural abnormalities are observed. The arrow indicates the giant marker. The zebra pattern is deduced from complementary FISH analysis with whole chromosome painting and locus-specific probes and corresponds to co-amplified sequences from chromosomes 10 and 12. This co-amplification was also observed on additional marker chromosomes (M). **(D)** Individual dye spectra, inverted DAPI, SKY spectrum and SKY false color of representative large markers selected from 4 metaphase cells (D1 to D4) (out of the 10 metaphase cells analyzed). Each dye spectra is identified by a lower case letter: a, b, c, d, e.

Figure 3. Array-Comparative Genomic Hybridization profile (aCGH). **(A)** Genome-wide copy number alterations in 95T1000 cells. The x axis represents chromosome 1 to chromosome X, and from pter to qter. The estimated-relative-copy-number (ECN) is on the y axis. The color gradient from blue to red corresponds to an ECN of 2 (blue) to high level of amplification (red). Discrete regions of gain are observed on chromosomes 4, 13, 17 and 20; and loss on chromosome 20. Large amplicons, and multiple focal amplifications are observed on chromosome 10 p (B) and 12 q (C). Key genes and loci for which fluorescence *in situ* hybridization (FISH) had been performed are noted.

Figure 4. Characterization of chromosomal imbalances and structural rearrangements by fluorescence *in situ* hybridization (FISH) on 95T1000 metaphase cells at passage P80. **(A-C)** Co-amplification of chromosome 10 and 12 regions. Co-localization of both green and red signals on the central region of the giant marker (arrow), as well as several other marker chromosomes (arrowheads), using Whole Chromosome Painting (WCP) probes for chromosome 10 (A) and 12 (B). Co-amplification of the *MDM2* (green) and RP11-269B1 (*NEBL* 10p12.31) probes (red) on the giant marker (arrow) and on several other marker chromosomes (C). Apparently normal chromosomes 12 and 10 were also identified (*). **(D)** High amplification of *MDM2* (green signals) located on one giant marker (arrow) and several marker chromosomes. Four apparently normal chromosomes 12 (*) identified by centromere 12 (red signals) and *MDM2* at their expected position were also observed. **(E)** Hybridization with the RP11-177H22 probe containing *MLLT10* (10p12.31) shows a high level of amplification on the giant marker (arrow) and several marker chromosomes. **(F)** Hybridization with the dual-color probes that flanks the 5' (RP11-30I11-green) and 3' (RP11-118B13-red) regions of *HMGA2*. High-level amplification of the 3' region of *HMGA2* on the giant marker and marker chromosomes is observed. In contrast the 5' region is not amplified.

Table 1. Fluorescence in situ hybridization (FISH) probes used in this study

Probe name	Locus/Gene or marker (alias)	¹ Genome coordinates
² TelVysion™ 12p/12q	12pter/8M16-SP6, 12qter/VIJ2yRM2196	
³ CTD-3109K12	12q13.3/5' <i>R3HDM2</i> (<i>INHC</i>)	chr12:56,048,383-56,074,880
⁴ RP11-16M7	12q14.2-12q14.3/ <i>GNS</i>	chr12:63,300,098-63,460,890
⁴ RP11-20P1	12q14.3/3' <i>WIF1</i>	chr12:63,571,935-63,727,652
⁴ RP11-109N1	12q14.3/5' <i>WIF1</i>	chr12:63,852,375-64,025,207
⁴ RP11-30I11	12q14.3/5' <i>HMG A2</i>	chr12:64,178,505-64,349,708
⁴ RP11-118B13	12q14.3/3' <i>HMG A2</i>	chr12:64,644,968-64,789,255
⁵ ZytoLight® SPEC MDM2, CEN 12	12q14.3-12q15/ <i>MDM2</i> , 12cen/D12Z3	chr12:67,488,247-67,520,481
² TelVysion™ 10p/10q	10pter/GenBank Z96139, 10qter/D10S2290	
⁴ RP11-150J3	10p13/ <i>FRMD4A</i>	chr10:14,125,465-14,277,321
⁴ RP11-269B1	10p12.31/ <i>NEBL</i>	chr10:21,093,656-21,254,619
⁴ RP11-34I5	10p12.31/ <i>NEBL</i>	chr10:21,413,645-21,559,356
⁴ RP11-177H22	10p12.31/ <i>MLLT10</i>	chr10:21,886,025-22,026,020
⁴ RP11-573G6	10p12.31/ <i>COMMD3, BMI1, SPAG6</i>	chr10:22,573,927-22,712,022
⁴ RP11-63G10	1p32.1/ <i>JUN</i>	chr1:58,951,678-59,094,341
⁴ RP3-325F22	6q23.3/ <i>MAP3K5</i> (<i>ASK1</i>)	chr6:136,892,189-137,046,976
² AneuVysion® LSI 13, LSI 21	13q14.2/ <i>RB1</i> , 21q22.13- 21q22.2/D21S259-D21S341- D21S342	
⁴ RP11-313L9	13q33.3-13q34	chr13:109,059,438-109,253,923
³ CTD-2328A22	13q34	chr13:109,925,937-110,037,953
² LSI CCND1, CEP11	11q13/ <i>CCND1</i> , 11cen/D11Z1	
² CDKN2A/ CEP9	9p21/ <i>CDN2A</i> , 9p11-q11	
² UroVysion®	3cen/D3Z1, 7cen/D7Z1, <i>CDKN2A</i> /9p21, 17cen/D16Z1	

¹Genome browser (www.genome.ucsc.edu) version Mar. 2006; ²Abbott Molecular, Des Plaines, IL, USA; ³Invitrogen, Carlsbad, CA, USA; ⁴Children's Hospital Oakland Research Institute (<http://bacpac.chori.org/>); ⁵Zytovision GmbH, Bremerhaven, Germany.

chr: chromosome

Table 2. Clonal structural rearrangements other than the large marker detected by SKY analysis.

Abnormal chromosome designation	Cell (modal number)										Composition
	1 (79)	2 (79)	3 (79)	4 (84)	5 (83)	6 (62)	7 (66)	8 (89)	9 (84)	10 (82)	
A	++	+	++	++	+	+	+	++	+	++	der(8)(8;4;8)x2
B	+	+	+	+	+	none	none	+	+	+	der(20)(12;13;12;20;12)
C	+	+	+	+	+	none	none	+	+	+	der(12)(12;15;12;11)
D	none	none	+	+	+	+	+	+	+	+	der(5)(5;3;16)
E	+	+	+	+	+	none	+	none	+	none	der(22)(22;1)
F	none	+	++	++	+	none	+	+	+	++	der(3)(3;6)x2
G	+	+	+	+	+	none	+	none	+	+	der(3)(3;12;18)
H	+	++	++	+	+	++	none	++	++	++	der(14)(X;14)x2
I	+	+	+	none	+	+	none	+	+	+	der(9)(9;1)
J	++	+	++	+	+	+	++	++	++	++	der(6)(6;12)x2
K	none	none	none	none	+	+	none	+	+	none	der(13)(19;13;8)
L	+	+	+	none	none	+	+	+	+	+	der(15)(8;15)
M	none	none	none	none	none	none	+	+	none	+	der(4)(4;13)
N	none	+	+	+	+	+	none	+	+	none	der(11)(11;4)
O	+	none	+	+	++	+	++	+	++	+	der(12)(12;3)

+ and ++ : one (+) or two (++) derivative chromosome/cell

Table 3: Characteristics of the chromosome segmental gains/ amplifications identified by array-comparative genomic hybridization.

Chromosomes	Number of segment(s)	Minimum SL/ECN	Maximum SL/ECN	Average SL/ECN	Amplicon's total length	Total size
Gains						
4p16.3-q27	8	0.4 Mb / 3	20.7 Mb / 3	6.2 Mb / 3	50 Mb	139 Mb
13q12.11-q34	4	0.1 Mb / 3	3.2 Mb / 7	1.6 Mb / 6	6.6 Mb	96 Mb
17q11.1-q25.3	10	0.2 Mb / 3	5.5 Mb / 8	1.1 Mb / 5	11.8 Mb	56 Mb
High level amplifications						
10p14-p11.21	45	30 kb / 3	0.7 Mb / 56	0.5 Mb / 22	7.6 Mb	27 Mb
12q12-24.31	67	70 kb / 3	2.4 Mb / 57	0.4 Mb / 18	27.8 Mb	82 Mb

SL: segment length; ECN: estimated relative copy number

Table 4: Real time quantitative RT-PCR.

Gene (exon)	Genomic locus	Fold change*
p14ARF	9p21.3	5.5
p16INK4a	9p21.3	22
TSPAN31	11p15.5	42
CCND1	11q13.2	5
DDIT3	12q13.3	2.5
GLI-1	12q13.3	0.6
CDK4	12q14.1	39
HMGA2 (1-2)	12q14.3	317
HMGA2 (3-4)	12q14.3	64
HMGA2 (4-5)	12q14.3	66
WIF1	12q14.3	1733
MDM2	12q15	48
YEATS4	12q15	83
RB1	13q14.2	1.2

*Values from normal adipose tissue are used as reference.