Characterization of the new human pleomorphic undifferentiated sarcoma *TP53*-null cell line *mfh-val2*

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Abstract Pleomorphic undifferentiated sarcoma (PUS), also called malignant fibrous histiocytoma, is a soft tissue sarcoma which occurs predominantly in the extremities. Its origin is a poorly defined mesenchymal cell, which derives to histiocytic and fibroblastic cells. The patient, a 58 year-old man, presented a lesion located in the forearm composed by spindle cells and multinucleated giant cells, which expressed vimentin and adopted a histological pattern formed by irregular-swirling fascicles. Cells were cultured in vitro and a new cell line was established. We characterized this new cell line by histological analyses, cytogenetics (using G-bands and spectral karyotype technique) and cytometric analyses. Cells were grown in culture for more than 100 passages. They had elongated or polygonal morphology. The cells presented a saturation rate of 70,980 cells/cm², a plating efficiency of 21.5% and a mitotic index of 21

mitoses per field. The cell line was tumorigenic in nude mice. The ploidy study using flow cytometry revealed an aneuploid peak with a DNA index of 1.43. A side population was detected, demonstrating the presence of stem and progenitor cells. Cytogenetics showed a hypotriploid range with many clonal unbalanced rearrangements. Loss of p53 gene was evidenced by MLPA. We describe, for the first time, the characterization of a new human PUS *TP53*-null cell line called *mfh-val2*. *Mfh-val2* presents a wide number of applications as a *TP53*-null cell line and a great interest in order to characterize genetic alterations influencing the oncogenesis or progression of PUS and to advance in the biological investigation of this tumor.

Keywords Cell line · Pleomorphic undifferentiated sarcoma · Malignant fibrous histiocytoma · *TP53*-null

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Introduction

Pleomorphic undifferentiated sarcoma (PUS), is a malignant neoplasm of uncertain origin that arises both in soft tissue and bone. The actual WHO-Classification uses the term of PUS or PUS NOS (not otherwise specified) and malignant fibrous histiocytoma (MFH) synonymously, but it tends to replace MFH, as PUS provides an accurate description of the tumor without implying the origin of the tumor cells (Fletcher 2006; Al-Agha and Igbokwe 2008).

PUS is a diagnosis of exclusion in case in which ancillary methods fail to demonstrate a specific line of differentiation. They account for 5% of all sarcomas in adults, most commonly occurring as deep seated lesions in the extremities in the elderly (Salo et al. 1999). Distant metastases appear in one-third of the cases, involving mainly lung, bone and liver (Salo et al. 1999; Zagars et al. 2003). Generally, there is immunohistochemical positivity only for vimentin and sometimes in a few cells for smooth muscle actin. PUS exhibits a heterogenous histology of spindle cells in a characteristic storiform pattern with pleomorphic and giant tumor cells. While the histological origin is unclear, it has been postulated that the tumor derives from histiocytes that morphologically and functionally differentiate to form fibroblasts (Matushansky and Maki 2005).

Cytogenetics has long been used to divide sarcomas into two broad groups: sarcomas with specific genetic alterations and sarcomas with complex unbalanced karyotypes (Mertens et al. 2002). Sarcomas with complex karyotypes are characterized by extensive cytogenetic abnormalities; these include deletions, amplifications, derivative chromosomes, and aneuploidy (Sandberg 2002; Mandahl et al. 1988). Tumor progression in both, simple and complex karyotype sarcomas are commonly associated with additional mutations in known cell cycle regulatory pathways (*RBI*, *TP53*, and/or their upstream/downstream targets).

Cancer stem cells are a subpopulation of self-renewing cells which show more resistance to chemotherapy and radiation therapy and facilitate invasion, recurrence and metastasis in cancer (Cojoc et al. 2015). Given their important role, cancer stem cells are postulated to be one of the main targets in the treatment of cancers. Tumor protein p53, is encoded by the tumor suppressor gene *TP53*, located at 17p13, and promotes DNA repair and, under certain conditions, apoptosis (reviewed in Ozaki and Nakawagara 2011).

In the present study, we report the characterization of a novel human cell line designated *mfh-val2*, derived from a human PUS. There are some established PUS cell lines, two of them presenting, like the present case, alterations in *TP53* (Fang et al. 2002; Hakozaki et al. 2006). However, *mfh-val2* presents a *TP53* deletion which has never been described before in a PUS cell line. Moreover, the existence of stem and

progenitor cells, present in our cell line, has not been investigated in previously reported PUS cell lines. To this extent, *mfh-val2* can be a useful tool to increase our knowledge about the biology of this tumor and to design new cancer therapeutics for the elimination of cancer stem cells.

Material and methods

Case report

A 54-year-old man was referred to the University Clinic Hospital of Valencia complaining of pain in the left forearm. Computed axial tomography showed a $7 \times 6 \times 4.5$ cm tumor. There was no evidence of metastatic disease at the moment of diagnosis. A biopsy was made, establishing a WHO diagnosis of grade III pleomorphic undifferentiated sarcoma. Tumor material was collected to perform the histopathologic characterization, and the molecular-genetic studies. Neoadjuvant therapy consisted in six cycles of 75 mg/m^2 doxorubicin, but the tumor recurred at 39 months after diagnosis. Approval for the study herein reported was provided by Institutional Ethics Committee of the University of Valencia and Clinic Hospital of Valencia (*Ley 14/2007 de Investigación Biomédica*, ethics committee approval on 2015/06/03).

Cell culture and establishment of cell line

Tumor tissue from the first recurrence was dissected under aseptic conditions. Cell culture was established as previously described (Gil-Benso et al. 2003, 2012): tissue was finely minced and the fragments were disaggregated using 0.02% collagenase type II (Sigma, St. Louis, MO, USA) in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% L-glutamine, 200 mM penicillin (50 U/mL), and streptomycin sulfate (50 $\mu\text{g/mL}$) (all from Gibco BRL, Grand Island, NY, USA) and incubated at 37°C for 20 min. The cells were seeded into 25-cm² tissue culture flasks (Nunc, Roskilde, Denmark) and incubated at 37°C in a humidified atmosphere with 5% CO₂. Subcultures were made using 0.25% trypsin-1 mM EDTA (Gibco BRL). The cultured cells were observed daily using an inverted microscope Zeiss Axiovert 135 (Carl Zeiss Vision, Oberkochen,

Germany). Photographs were taken using $\times 10$ and $\times 20$ objectives. Cells were stored under liquid N_2 in culture medium containing 5% dimethylsulfoxide (Sigma).

Growth kinetics

Growth kinetics were determined by trypsinizing the cultures in triplicate and resuspending the cells in medium. The viable cells were counted in a hemocytometer by dye exclusion with 0.1% trypan blue in PBS every 24 h for 4 days and at day 7. A growth curve was performed (Fig. 1c). Doubling time of the cell population was estimated in a logarithmic growth phase, using the formula: doubling time = $\ln(2)/\text{growth rate}$, where growth rate is equal to $1/\text{time} \times \ln(\text{cell number at time zero}/\text{cell number at time } t)$.

Cell density and plating efficiency

Using cells from the 25th passage, 10^5 cells were seeded in a 25-cm² tissue culture flask. The formation of a confluent cell layer was confirmed as taking approximately 2 weeks. Cells were isolated by trypsinization and counted, from which cell density and saturation density were calculated. 5×10^2 cells were seeded in an 80-cm² culture flask and cultured for 15 days. The medium was aspirated and the cells were rinsed with PBS, fixed in 95% ethanol, and stained with hematoxylin. Plating efficiency was calculated as described elsewhere (López et al. 2016). Briefly, cells were plated at a low density and their plating efficiency was determined after overnight culture according to the following formula: $100 \times \text{number of colonies counted}/\text{number of seeded cells}$. Mitotic cells were detected and mitotic index was calculated.

Establishment of clones

A single-cell cloning by the limiting dilution method was performed using cells in the 25th passage as previously described (Gil-Benso et al. 2003). Briefly, cells were resuspended in complete medium, and diluted to a density of 50 cells/mL. Twenty microliters of the cell suspension was plated in each well of a 96-well plate polystyrene (Nunc). The colonies formed were subsequently trypsinized and seeded in

35-mm dishes. Three clones named *Viriato*, *mfh-val2-B7* and *mfh-val2-C1* were analyzed.

Histological and immunohistochemical characterization

The surgical sample was fixed in 10% formaldehyde, paraffin embedded and stained with hematoxylin and eosin. Immunohistochemistry by the indirect peroxidase method was performed on primary tumor, recurrence and xenografts. Antigen retrieval was performed with heat-induced epitope retrieval with autoclave at 1.5 atmospheres for 3 min in citrate buffer solution. Negative controls were applied by substitution of the primary antibody with PBS alone. The monoclonal antibodies used were directed against vimentin, desmin, smooth muscle actin, S-100, AAT, CD117, H-caldesmon, CD68, CD34, CD31, and CK AE1/A3 (All from DAKO, Carpinteria, CA, USA).

Immunocytochemistry

Mfh-val2 cells were grown in chamber slides, rinsed with PBS, fixed in cold methanol acetone for 5 min and air dried for 20 min. The antibodies applied were the same that in immunohistochemical studies. Cells reacted with each of the primary antibodies for 1 h at room temperature. The attached antibodies were visualized by an avidin–biotin–peroxidase procedure (DAKO).

Tumorigenicity assay

Balb/c nude mice (Charles River Laboratories, Wilmington, MA, USA) were bred and maintained in specific pathogen-free conditions at the animal production service facilities of our institution (University of València). All assays involving mice were approved by the Institutional Animal Care and Use Committee (Universitat de València). The endpoint of the tumorigenicity assay was elucidating whether *mfh-val2* cells were able to generate a tumor mass in vivo. Suspended *mfh-val2* cells were washed with PBS, and 1×10^6 cells were subcutaneously injected into each of three immunodeficient 4–6-week-old male Balb/c nude mice. Mice health and behavior was monitored daily during assay. When detectable, tumor dimensions were measured and mice were sacrificed by cervical dislocation. Tumor volume was calculated, as

Fig. 1 Characterization of the tumor, cell line and xenografts. **a** Histological study of the tumor tissue. The sample was stained with hematoxylin and eosin. **b** Cell culture from the tumor. Phase-contrast image of the cell culture. **c** Doubling time. The number of cells in the culture were represented against the time in hours. The time required for duplicating the number of cells was estimated in a logarithmic growth phase. **d** *Viriato* cells. Phase-contrast image of the cell clone. **e–g** Immunocytochemical studies in *mflh-val2* cultured cells. Vimentin, smooth muscle actin and S-100 protein expression was evidenced in *mflh-val2* fixed cells. **h** Histological study of the xenografts. The xenografts were stained with hematoxylin and eosin. **i–j** Immunohistochemical studies in xenografts. Cells from the xenografts expressed Ki-67 and vimentin

described elsewhere, with the formula: $V = L \times l^2 \times 0.5$, where L and l represent the larger and the smaller tumor diameters, respectively.

Flow cytometry

Ploidy study

Cells were harvested, fixed with cold 70% ethanol and stained with propidium iodide (50 µg/mL) containing RNase A (1 µg/mL) (Sigma). Human peripheral blood lymphocytes from a male donor were used as control for diploid cells. Lymphocytes were isolated from whole blood using Lymphoprep separation medium (Nycomed Pharma AS, Oslo, Norway). Measurement of at least 10,000 nuclei was made using an Epics Elite cell sorter (Coulter Electronics Inc., Hialeah, FL, USA). Histogram data were analyzed with Mcycle software (Phoenix, AZ, USA). The ploidy level was expressed as the DNA index, assigning the value 1.0 to diploid control cells. Lymphocytes from healthy donors were used as references and the procedure was performed following the manufacturer's instructions.

Side population (SP)

Cells growing in exponential phase were resuspended in complete medium (10^6 cells/mL) and stained with 5 µg/mL of Hoechst 33342 for 90 min at 37 °C, with or without the inhibitor of ABCB1 (50 µM verapamil, Sigma), in the presence of propidium iodide at 5 µg/mL to exclude dead cells. Detection of the side population fraction cells was performed with a MoFlo cell sorter (Beckman-Coulter, Brea, CA, USA).

Conventional cytogenetics

Cells obtained at seven days of short-term culture of the tumor sample and from passages 15 and 30 of the cell line were exposed to 0.02 µg/mL Karyo Max® Colcemid® (Gibco BRL) for 2 h and harvested routinely. Metaphase chromosomes were GTG-banded by trypsin–Giemsa technique and karyotyped according to the International System for Human Cytogenetic Nomenclature, 2013 (Shaffer et al. 2013).

Spectral karyotype study (SKY)

Slide preparation and hybridization using commercial probes were carried out according to the manufacturer's instructions (Applied Spectral Imaging, Migdal Ha'Emek, Israel). Images were acquired using an ASI SD300 Spectracube mounted on a Zeiss Axioplan 2 microscope (Carl Zeiss Vision).

Molecular analysis

Amplification for *CDK4*, *Cyclin D1*, *MFHAS1*, *MDM2* and *MYCN* was determined by differential PCR using two pairs of primers, one corresponding to the target gene and the other to an internal control; the dopamine receptor gene (*DRD2*) was used for *MDM2*, the β-globin gene for *CDK4*, *MFHAS1* and *MYCN*, and the gamma-interferon gene (*IFNG*) for *Cyclin D1* analysis (all the primers from: Life Technologies Inc., Barcelona, Spain). PCR was performed with the primers CATG TAGACCAGGACCTAAGG and GGAGGTCGGTAC CAGAGTG for *CDK4* (57 °C, 33 cycles), TTCTGC CTTTGATGTTAC and AGGCTGAATCAATGTCTT for *Cyclin D1* (50 °C, 30 cycles), GCATTTCCAGCCA CCTCAG and ACAGAAAGCCCTCCACATAC for *MFHAS1* (58 °C, 30 cycles), GTCAGCTAAGGAAA TTTCAGG and TCGACCTTGACAAATCACAC for *MDM2* (57 °C, 35 cycles), and AGTTCCTTCCACC CTCTCCT and CACCCAGCAACCCCCTAAAC for *MYCN* (59 °C, 30 cycles). Amplified positive, negative and water controls were used in each reaction. Multiplex Ligation-dependent Probe Amplification (MLPA) was performed for the detection of copy number changes of *TP53*, *EGFR*, *ERBB2*, *CDKN2A p14ARF* and *CDKN2A-p16* with SALSA MLPA probemix P105-C2 (MRC-Holland, Amsterdam, The Netherlands). Methylation specific-MLPA for the detection of methylation status of genes located in the *CDKN2A–CDKN2B*

(p14ARF–p16INK4A–p15INK4B) *loci* at 9p21.3 was performed with SALSA MLPA ME024-B1 9p21 CDKN2A/2B region probemix (MRC-Holland).

Short tandem repeat (STR) analysis

The analysis of STR markers was performed by Genomic Service from Biomedical Research Institute “Albert Sols” (CSIC-UAM, <https://www.iib.uam.es/portal/web/genomica>) using genomic DNA extracted from *mfh-val2* and from patient’s primary tumor. This experiment was conducted using GenePrint® 10 System (Promega, Darmstadt, Germany) according to the manufacturer’s instructions and included ten human loci: D5S818, D13S317, D7S820, D16S539, VWA, TH01, Amelogenin, TPOX, CSF1PO and D21S11, for human cell line authentication. Amplification products were evaluated by an ABI 3130XL Genetic Analyzer (Applied Biosystems, Weiterstadt, Germany) and GeneMapper software (Life Technologies GmbH, Darmstadt, Germany).

Mycoplasma detection

To discard mycoplasma contamination, *mfh-val2* cells were stained with Hoechst 33342 (5 µg/mL) (Sigma) for 10 min at 37 °C and examined with a fluorescence microscope Zeiss Axioplan 2 (Carl Zeiss Vision, Oberkochen, Germany) at ×1000 under oil immersion.

Statistical analysis

Statistical differences were determined using the Mann–Whitney *U* test for groups of independent samples. Data were analyzed with GraphPad Prism 6 software. Significance was accepted at least at **P* < 0.05 level.

Results

Histological and immunohistochemical studies

The histological study with hematoxylin–eosin showed a mesenchymal primary tumor formed by elongated irregular eosinophilic cells with anaplastic nuclei adopting a fascicular swirling pattern. The tumor also presents areas of hyalinization, abundant

mitoses and some multinucleated giant cells (Fig. 1a). The number of mitosis was greater than 10 in 10 fields (40×), being the majority atypical. There were areas of anaplasia and necrosis. Immunohistochemical studies showed expression of vimentin, and focally for smooth muscle actin and S-100 protein. AAT, CD117, H-caldesmon, CD68, CD34, CD31, CK AE1/A3 and desmin proved negative. On the basis of these findings, PUS grade III was diagnosed. The recurrence presented the same patterns of expression markers described for the primary tumor.

Establishment of the cell line and phase-contrast morphology

The first passage of the cell line was performed at day 9 from seeding. Cells were passaged routinely in vitro more than 100 times. It took around 51 weeks from the establishment of the primary culture to the 100th passage (2 passages/week). Cells showed a slightly elongated polygonal morphology and grew forming clusters and showing a storiform arrangement (Fig. 1b) associated with giant multinucleated cells. Cultured cells had a saturation density of 70.980 cells/cm² and a plating efficiency of 21.5%. The mitotic index was 21 mitosis per 10×-field. A growth curve was conducted and the doubling time was calculated in the exponential phase, with a value of 56 h (Fig. 1c). According to the growth data, 230 population doublings were estimated to occur from the establishment of the primary culture to the passage No. 100th, approximately. After single cell cloning, multiple clones were obtained; three cell lines showing similar phenotypes were analyzed: *Viriato*, *mfh-val2-B7*, and *mfh-val2-C1* (Fig. 1d shows *Viriato*).

Immunocytochemical characterization

Mfh-val2 cells and *Viriato* clonal cells were positive for vimentin (Fig. 1e), positive in some cells for smooth muscle actin (Fig. 1f) and S-100 protein (Fig. 1g) and negative for AAT, CD117, H-caldesmon, CD68, CD34, CD31, CK AE1/A3 and desmin.

Heterologous transplantation to nude mice

The injection of *Mfh-val2* cells into congenitally athymic mice induced a visible solid tumor at the site

of inoculation after 10 weeks. The tumor presented, microscopically, the same morphological characteristics of the original tumor. The cells retained their proliferative activity. There were intratumoral blood vessels and some necrotic areas, as seen with hematoxylin–eosin stain (Fig. 1h). Cells from the xenografts were positive for the proliferation marker Ki-67 (Fig. 1i) and vimentin (Fig. 1j).

Flow cytometry

A DNA index of 1.43 corresponding to a hypotriploid population was obtained from the primary and recurrent tumor using human male lymphocytes as ploidy control. *Mfh-val2* cells at passage 25 had a DNA index of 1.43 (Fig. 2a). The xenograft obtained by inoculation of *mfh-val2* cells showed a peak corresponding to a 1.45 DNA index. Flow cytometry analyses also evidenced the presence of a side population of stem cells in *mfh-val2*, which was abolished in presence of verapamil (Fig. 2b).

Cytogenetic studies

Tumor Twenty-five cells in the hypotriploid range were counted; the modal chromosomal number was 65. By conventional cytogenetics many clonal unbalanced structural anomalies were detected, affecting chromosomes: X, Y, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 16, 17, 18, 21 and 22 (data not shown).

The spectral karyotype of the cell line at the 35th passage (Fig. 2c), showed 34 chromosomal rearrangements, all of them unbalanced, affecting the same chromosomes above cited.

The representative karyotype was: 57~69, +del(X)(p12),der(Y?)t(Y?;7)(q11.2;q?), +der(Y?) t(Y?;4)(p11.2;?), +der(1)del(1)(q21)t(1;6)(p22;?), +der(1)del(1)ins(1;10)(p22;q?), del(2)(p12),del(2)(q12),del(3)(p12q12)x2,der(4)t(4;11?)(p10;?), +der(4)dup(4)(q12;q26) t(4;15)(p10;q?), +5,del(5)(p12),der(5)del(5)(q12)t(2;5)(?;p15),del(6)(p12q12),-7, del(7)(p12),del(8)(p12), +der(8)del(8)(p12) t(3;8)(?;q24),del(9)(p12), +i(9)(q10), del(9)(p12q12), +der(9)t(9;10;22?)(q21;?;?),del(10)(q11.2)x2, +der(10) t(3;7;10;17;19;22)(?;?;p12;q?;?;?),del(11)(p12),i(12)(q10), +i(12)(q10), der(14) t(10;14)(?;q32), +der(14)dic(Y?;14)(p11.2;p11.2),del(16)(p12),der(16)t(14;16) (q?;q22), del(17)(p12),der(17)t(6;17)(q?;q25), +der(17)t(17,18)(q11.

2;?), +del(18)(q12), +19, +19, +19, +20, +der(21)t(3;21)(?;p11.2),der(22)t(10;22),(?;q11.2) [cp10].

Molecular results

No gene amplification for *CDK4*, *Cyclin D1*, *MFHAS1*, *MDM2* or *MYCN* was found by PCR. Allelic status of *EGFR*, *ERBB2*, *CDKN2A* and *CDKN2B* (p14ARF–p16INK4A–Targeting mutant p53 in human tumor sp15INK4B) determined by MLPA was normal, but not the *TP53* status. Analyses of p53 gene by MLPA in the fresh tumor showed a quantification of 0.2 relative fluorescent units (RFU) in every *TP53* exon plus 0.4 additional RFUs for exons 1A and 1B. In the *mfh-val2* cell line, approximately 0.8 RFUs were quantified for exons 1A and 1B, with no detection of fluorescence for exons 2 to 12 (Fig. 3a). RFU differences between the “positive control” values and its counterparts in “tumor” and “cell line” groups were significant, as determined by Mann–Whitney *U* test. No hypermethylation in *CDKN2A–CDKN2B* region was detected.

Short tandem repeat (STR) analysis

The analysis of STR markers was performed to verify the origin of the cell line from the tumor. The frozen primary parental tumor tissue and *mfh-val2* cell line showed the same STR profile at all the markers assayed. This analysis demonstrated that *mfh-val2* cell line originated from the patient. STR profile of these cells did not correspond to any of the collected cell lines in the database of ATCC (Table 1).

Mycoplasma detection

Mfh-val2 cultured cells at passages 20 and 40 stained with Hoechst 33342 did not show mycoplasma contamination.

Discussion

We have established a novel cell line, *mfh-val2*, derived from a local recurrence of a PUS. PUS is a high grade soft tissue sarcoma of adult life, often presenting as an aggressive, large, sarcoma of the extremity (Salo et al. 1999; Zagars et al. 2003). The 5-year survival is of 74%, the distant recurrence rate is

Fig. 2 Flow cytometry and cytogenetic studies. **a** *Mfh-val2* cells ploidy by flow cytometry. DNA histogram shows one control peak corresponding to human male lymphocytes DNA content (DNA index = 1). The other peaks and valley correspond to DNA content of *mfh-val2* cells in the different cell cycle phases (DNA index = 1.43). The experiment was performed three times, with cells from different passages. The DNA histogram is representative from the three independent experiments. **b** Presence of a side population in *mfh-val2* by flow

cytometry. The *dot plot* shows Hoechst blue fluorescence against Hoechst red fluorescence of *mfh-val2* cultured cells. Presence of 50 μ M verapamil abolished the side population (*right*). The experiment was performed three times, with cells from different passages. *Dot plots* are representative from the three independent experiments. **c** Cytogenetic analysis of *mfh-val2* cells by spectral karyotyping technique and representative karyotype formula

29% and the local recurrence rate is 20% (Zagars et al. 2003). Although these studies span several decades, their outcomes are similar. The tumor showed a histiocytic phenotype, with cells rearranged in a fascicular swirling histological pattern. The tumor cells showed expression of vimentin, and focally for smooth muscle actin and S-100 protein. The cultured cells also showed expression for these markers. They have a spindle or polygonal shape, similar to other pleomorphic PUS cell lines previously reported (Fang et al. 2002; Nakatani et al. 2001; Nishio et al. 2003).

Mfh-val2 was tumorigenic in athymic nude mice, forming tumors with histopathologic features similar to those of the original tumor.

Cytogenetically, PUS is characterized by multiple, often complex chromosome abnormalities (Sandberg 2002; Mandahl et al. 1988; Schmidt et al. 1998). No common characteristic aberration could be distinguished. Recurrent breakpoints are seen, particularly in chromosome bands 1p36, 1q11, 1q21, 3p12, 11p11, 17p11 and 19p13 (Choong et al. 1996; Fletcher et al. 2002). In line with this finding, karyotypic complexity

Fig. 3 MLPA analyses of p53 gene status in tumor cells and *mfh-val2* cells. **a** *TP53* dosages were calculated by MLPA in stromal cells (“positive control”), tumor cells (“tumor”) and *mfh-val2* cells (“cell line”). Exons 1A, 1B, 2, 4, 5, 6, 8 and 12 were analyzed. Values are expressed in relative fluorescent units (RFU). Values under 0.2 RFU represent a total loss of *TP53* exons copies, values between 0.2 and 0.8 represent the presence

of one copy of the exons and values over 0.8 represent two copies of the exons. Results are expressed as mean $\pm$ S.E.M., from four independent experiments for each kind of cells. ** $P < 0.01$ and *** $P < 0.001$ levels (Mann–Whitney U test) for each variable with respect to its “positive control” value. **b** Diagram with the hypothetical p53 gene status in the three cases according to the MLPA results

was found to be a strong prognostic factor (Mertens et al. 2002). *Mfh-val2* cell line presents the main genetic characteristics of PUS with a hypotriploid chromosome range and many clonal chromosome abnormalities being all of them unbalanced; all above-mentioned chromosomes were implicated. Furthermore, a multiple rearranged chromosome between chromosomes 3, 7, 10, 17, 19 and 22 was found, showing a great degree of chromosomal breakage and loss of DNA repair. Interestingly, chromosome 9 was highly rearranged, as shown by SKY analyses and supported by conventional cytogenetics. No hypermethylation was detected in *CDKN2A–CDKN2B* region.

MLPA analyses of p53 gene in the fresh tumor and in *mfh-val2* cells (Fig. 3a), prompted us to hypothesize that: (1) there was a fraction of the tumor cells with two copies of *TP53* in the fresh tumor, which can be related to the value of 0.2 RFUs detected in all the exons; (2) The subtraction between 0.6 and 0.2 value in exons 1A and 1B may correspond to the presence of a single copy of those exons in a different fraction of the tumor cells; (3) the fluorescence levels in *mfh-val2* cells indicate the only existence of two copies of exons 1A and 1B from p53 gene in the nuclear genome. These results can be explained in the fresh tumor by the complete loss of one *TP53* allele and a partial deletion of the sequence in a second allele, whereas the

Table 1 STR analysis of primary tumor and *mfh-val2*

	Primary tumor	<i>mfh-val2</i>
D5S818	12	12
D13S317	11	11
D7S820	9, 13	9, 13
D16S539	11	11
VWA	15	15
TH01	9.3	9.3
Amelogenin	x, y	x, y
TPOX	8	8
CSF1PO	11	11
D21S11	28, 31.2	28, 31.2

Comparison between STR markers of primary tumor cells and *mfh-val2* cells. The analysis included ten human loci: D5S818, D13S317, D7S820, D16S539, VWA, TH01, Amelogenin, TPOX, CSF1PO and D21S11

most suitable explanation for the result in *mfh-val2* cell line would be the duplication of the remaining *TP53* partially deleted copy, together with the negative selection of the cells with two copies of *TP53* in vitro (Fig. 3b). Our results demonstrate that despite the abundant amount of chromosome 17 material observed in the karyotype (Fig. 2c), there is not any complete copy of p53 gene present in *mfh-val2* cells genome. Thus, the *mfh-val2* cell line, lacking nine exons of each p53 allele, can be considered as a *TP53*-null cell line.

The tumor suppressor gene *TP53*, located at chromosome 17p13, is extensively known for its task in promotion of DNA repair, aging, genetic instability, cell cycle control and induction of apoptosis (reviewed in Meek 2015). Mutations in *TP53* are known to cause or participate in a variety of recognized human neoplasms (reviewed in Lehmann and Pietenpol 2012) and, from them, in approximately 30% of PUS tumors, without showing any specific site of presentation or “hot spot” (Hakozaki et al. 2006). To our knowledge, only two PUS cell lines have been described with *TP53* mutations (Fang et al. 2002; Hakozaki et al. 2006); the *GBS-1* cell line presented a missense mutation (TTC/TTA) in codon 312 of exon 7 of p53 gene. It is noticeable that this cell line also showed a loss of the normal p53 allele. This loss of heterozygosity could have occurred, according to the authors, during or after the tumor progression (Fang et al. 2002). The other PUS cell line, named *FPS-1*,

showed a nonsense mutation (CGA/TGA) in codon 213 of exon 6 (Hakozaki et al. 2006). The present cell line, *mfh-val2*, carries a homozygous partial deletion of the tumor suppressor gene. Lack of *TP53*, is critical for cell immortalization and, potentially, tumorigenesis (Hanahan 2000). Absence of *TP53* allows immortalization of cells by lacking of G₁ cell cycle checkpoint control (which implies increased cycling), and lacking time to repair DNA alterations, with subsequent accumulation of mutations (Dumble et al. 2002). Taken together, these findings suggest that inactivation of *TP53* by homozygous deletion may be important for pleomorphic PUS development and *mfh-val2* immortalization.

The hypothesis that cancer stem cells lead the tumorigenic process has received several consents in the last years. This hypothesis suggests that tumors are initiated and maintained by a subset of cancer cells capable of self-renewal and differentiation into bulk tumor cells (Chen et al. 2013). Stem cells are slowly proliferating cells that give rise to proliferating cells that eventually fully differentiate into mature tissue components. In cancer, cancer stem cells are involved in drug resistance, by expressing members of the ABC transporter family, and in the development, growth and recurrence of tumors (Moitra 2015).

Since it is well known that side population from tumors is enriched in cancer stem cells (Patrawala et al. 2005), this population was investigated in *mfh-val2* by flow cytometry. The presence of a side population demonstrated the maintenance of functional cancer stem cells in *mfh-val2*, and so, the usefulness of this cell line as an in vitro model of PUS.

The present article describes, for the first time in the literature, a PUS *TP53*-null cell line. The *TP53* null status was achieved by a dual devoid of both alleles, as checked by MLPA. To our knowledge, this specific deletion has never been reported previously, not only in a PUS cell line, but in any human tumor cell line. To date, the two human tumor cell lines with the most similar kind of deletion are H1299 lung carcinoma and Saos-2 osteosarcoma cell lines, in which the p53 gene is entirely deleted (Berglund et al. 2008).

TP53-null cell lines have a wide diversity of applications: these cell lines can be used as recipients to reintroduce either wild-type or mutant *TP53* in the study of the functional roles of p53 isoforms in cancer (Silden et al. 2013; Hafsi et al. 2013; Fritzsche et al. 2004), or as control cells in functional studies about

other members of p53 family like p73 (Holcakova et al. 2008; Stros et al. 2002; Zeng et al. 2000). TP53-null cell lines are also used as controls in pharmacological and biological studies of molecules that modulate the p53 pathway (Guo et al. 2012; Tanner and Barberis 2004; Li et al. 2008; Rui et al. 2004; Pise-Masison et al. 2001), and could be useful in studies of drugs whose treatment result is correlated with TP53 status in the tumor cells (Xu et al. 2016). TP53-deficient cell lines can be also used to study proteins related to p53 pathway: for instance, multiple connections between p53 and p14/p16/p15 pathways have been described (Efeyan and Serrano 2007). Most tumors without TP53 alteration show a deletion of p14 or lack of mRNA expression, suggesting that p14 loss could be an alternative genotype for direct TP53 inactivation (Pérot et al. 2010). Additionally, it has been described that cells with TP53 deficiency are less sensitive to p16-induced cell cycle arrest (Lowe and Sherr 2003). Therefore, *mfh-val2*, as a TP53-null cancer cell line may be a useful tool to study those related pathways. It has been described that many micro RNAs are under direct TP53 transcriptional control, like mir-34 family, mir-192 or mir-195, for example. The study of miRNAs expression profile in TP53-null cell lines could show if there is a difference between tumors according to their TP53 allelic status (Otsuka and Ochiya 2014). Moreover, *mfh-val2* is the first human TP53-null PUS described cell line and can be a promising tool in a variety of experimental procedures studying the p53 pathway, with specific genetic features which make it interesting for studies of this kind of cancer and having a population of cells with stemness features. Cancer cell lines have been the historical standard both for exploring the biology of human tumors and as preclinical models for screening potential therapeutic agents. Because of their genetic and biological characteristics, *mfh-val2* cell line could be interesting for the design of novel therapeutic strategies for PUS. Furthermore, this established cell line might represent a useful tool for the investigation of functional features of cancer stem cells.

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