Establishment and cytogenetic characterization of a cell line from a pulmonary metastasis of osteosarcoma

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Abstract Osteosarcoma (OS) is the most frequent malignant bone tumour in children and adolescents. In metastatic patients, the most common site of metastasis is the lung. There are relatively few cell lines of metastatic OS reported in the literature and the cytogenetic aspects of OS metastases are still controversial and inconclusive. Here we describe the establishment of a new OS cell line, M-OS, from a pulmonary metastasis of a typical osteoblastic OS of an 11-year-old boy with metastatic OS at diagnosis. M-OS cells have been maintained in culture for over 50 passages for more than 1 year. M-OS was characterized by immunohistochemistry, conventional

cytogenetics and fluorescence in situ hybridization (FISH). In order to evaluate in vitro cell modification, the immunohistochemical analysis was performed in three different moments of the cell line: 10th, 30th and 50th passages. The conventional cytogenetic analysis revealed the ploidy of M-OS cell line as near-diploid, with most metaphases hyperdiploid and tetraploid. We found a copy number gain of *MDM2* gene as the most frequent alteration in the FISH analysis. The immunohistochemical analysis confirmed that M-OS cell line maintained the osteogenic nature even after all passages for the cell line establishment in vitro.

Keywords Osteosarcoma · Lung metastasis · Cell line · Cytogenetics · FISH

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Introduction

Osteosarcoma (OS) is a highly metastatic malignant bone tumour and presents high incidence in children and adolescents (Mirabello et al. 2009). In Brazil, the presence of metastases at diagnosis was identified as the most significant single factor influencing OS outcome (Petrilli et al. 2006). The OS progression is characterized by increased metastatic spread, with high tendency to disseminate to the lungs and about 20 % of OS patients already present pulmonary metastases at diagnosis (Bacci et al. 2008).

Current strategies to treat metastatic OS are still limited, since there is little knowledge regarding the biological behavior of OS metastatic cells (Posthuma-DeBoer et al. 2011). In this sense, cell lines generated from metastatic OS could be useful in biological systems to better understand the mechanisms of migration and invasion of OS cells (Gillette et al. 2008). However, there is a limited number of OS cell lines established. Accordingly, the vast majority of studies in the literature describe the establishment of cell lines derived from OS primary tumours, which then generate metastatic sublines (Gillette et al. 2008; Khanna et al. 2000; Kimura et al. 2002; Chen et al. 2009). There is a cell line derived from a lung metastatic nodule of a chemical carcinogen-induced OS in a rat model (Honoki et al. 1998), but it has not been established yet a cell line derived directly from a lung metastatic nodule of a conventional human OS.

Here, we describe for the first time the establishment and cytogenetic characterization of a new human OS cell line derived directly from a pulmonary metastasis nodule of OS (M-OS). Also, immunohistochemical analysis was performed to confirm the osteogenic origin of M-OS cell line.

Materials and methods

Sample

The M-OS cell line was established from a pulmonary metastasis nodule of a typical osteoblastic OS from an 11-year-old boy with metastatic OS at diagnosis, admitted to treatment in the Pediatric Oncology Institute (IOP/GRAACC/UNIFESP). The standard histopathological information of this pulmonary metastasis was collected in order to confirm tumour content,

cell viability and exclude a necrotic sample. Accordingly, the sample of OS metastasis included in this study presented 100 % of tumour viability. The pulmonary nodule was obtained from one OS patient who underwent thoracotomy for the metastasis resection. This procedure was performed only after informed consent was signed by the parents of the patient, according to the Research Ethics Committee of the Federal University of São Paulo (CEP/UNIFESP no 0050), which stands that the use of the tumour specimen in the present study does not involve additional risks to the patient or the requirement of other procedures, ensuring complete anonymity of the patient.

Cell line establishment

The fragment of OS lung metastasis was disaggregated mechanically with scissors and enzymatically with collagenase type II 1,400 U/mL (Gibco Invitrogen, Carlsbad, CA, USA). Cells in suspension were incubated in 25 cm² culture flasks with RPMI 1640 media supplemented with 10 % fetal bovine serum and antibiotics (gentamicin sulfate at the final concentration of 500 µg/ml) at 37 °C with 5 % CO₂. The M-OS has been cultivated for more than one year and is presently in the 55th passage. Starting at the 5th passage, all passages of M-OS are cryopreserved in Recovery Cell Culture Freezing Medium (Gibco Invitrogen) at −80 °C. The morphological analysis of M-OS was performed for three different moments of the cell line: 10th, 30th and 50th passages, using an inverted microscope (Olympus CKX31, Olympus, Tokyo, Japan).

Conventional cytogenetics analysis

Standard culture and harvesting procedures for cytogenetic analysis were performed as previously described with modifications (Sandberg and Bridge, 1994). For the cytological preparations, M-OS cells from the 50th passage were exposed to KaryoMAX Colcemid (10 µg/ml) (Gibco Invitrogen) at a final concentration of 0.1 µg/ml, 2 h prior to harvest. Cells were harvested with trypsin TrypLE Express (Gibco Invitrogen), treated with hypotonic solution (KCl 0.0075 mol/L) for 15 min at 37 °C and fixed three times with methanol: glacial acetic acid (3:1). Slides were banded using standard trypsin Giemsa banding (GTG-banding). The number of chromosomes of 100 metaphase spreads was determined and 30 metaphases were karyotyped

according to the International System of Chromosome Nomenclature (Shaffer and Tommerup 2009).

Fluorescence in situ hybridization

Fluorescence in situ hybridization (FISH) was performed on recently made slides from methanol acetic acid fixed M-OS cells from the 50th passage. The DNA probes for FISH analysis included *RB1* deletion probe (LPS011) and *MDM2* amplification probe (LPS016) (Cytocell, Cambridge, UK). The *RB1* deletion probe presents a control probe in 13qter and the *MDM2* amplification probe has a centromeric control in 12cen. The FISH procedure was performed according to manufacturer's instructions. The slides were visualized by fluorescence microscopy, images captured using the ISIS software (Zeiss, Jena, Germany) and at least 300 interphase nuclei were analyzed for each gene.

Immunohistochemistry

Immunohistochemical characterization of M-OS cell line was performed for markers of proliferation (P53 and KI67), apoptosis (BCL2), inflammation (COX2), mesenchymal phenotype (VIMENTIN) and osteoblast (OSTERIX and OSTEOCALCIN). The immunohistochemistry procedure was performed on cell blocks prepared from M-OS cells. The cell block was fixed in formalin and routinely processed and embedded in paraffin. Sections (5 µm) were cut and used in the immunostaining procedure with the following primary antibodies: anti-VIMENTIN (clone V9, 1:300 dilution), anti-COX2 (clone CX-294, 1:100 dilution), anti-P53 (clone DO-7, 1:100 dilution), anti-KI67 (clone SP6, 1:250 dilution) (Dako, Carpinteria, CA, USA), anti-BCL2 (clone 124, 1:350 dilution) (Diagnosis Biosystems, Pleasanton, CA, USA), anti-OSTERIX (clone Y-21, 1:50 dilution) and anti-OSTEOCALCIN (clone FL-100, 1:50 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Results

Establishing M-OS cell line

The morphological analysis of M-OS in the 10th, 30th and 50th passages revealed that all of them present

similar spindle-shaped cell morphology and disorganized cell growth pattern (Fig. 1). The M-OS cell line was characterized by the presence of foci of cell clusters (Fig. 1b) and larger cells with cytoplasmic extensions (Fig. 1f). The doubling time of the different passages was approximately 48 h with 80 % confluence at 72 h. Only cells from the 10th passage presented faster growth with 80 % confluence at 48 h.

Cytogenetic analysis

The analysis of the chromosome number of M-OS revealed the ploidy of the cell line as near-diploid (55 %) and near-tetraploid (31 %) (Table 1). The conventional cytogenetic analysis of 30 metaphases revealed the most frequent chromosomal alterations as being numerical and only a reduced number of metaphases presented marker chromosomes, but they were not clonal. We could detect two cell populations in the M-OS cell line, one was near-diploid (37–57 chromosomes) with a modal number of 45 and the other was near-tetraploid (90–94 chromosomes) with a modal number of 90 (Fig. 2). Seven metaphases presented a diploid karyotype 46, XY and only one near-haploid metaphase was detected. The most common numerical alterations in all metaphases analyzed were the losses of chromosomes 9, 13, 14, 16, 17, 21, 22 and Y. We divided the composite karyotype of M-OS in diploid and tetraploid ranges. The composite karyotype of near-diploid population was 37–57, XY, −Y, −9, −13, −19, −21 [cp11]/46,XY[7] and the near-tetraploid 90–94, XXXY, −14, −16, −17, −22 [cp11].

The FISH analysis of *RB1* and *MDM2* genes showed that the most frequent alteration was the aneuploidy involving the chromosome 12. In this sense, a copy number gain of *MDM2* was found in 27 % of cells. The *RB1* homozygous deletion was found only in 1 % of cells and no *MDM2* amplification was detected.

Immunohistochemistry

The immunohistochemical analysis revealed similar positivity for the qualitative markers analyzed in all passages of M-OS cell line (Table 2). The qualitative immunohistochemical analysis included the osteoblast proteins OSTERIX and OSTEOCALCIN, the anti-apoptotic marker BCL2, the inflammatory modulator

Fig. 1 Morphology of M-OS cells under phase-contrast microscopy. M-OS cells at passage 10 (A10) with 100 % confluence, spindle-shaped cells, $\times 10$ (**a**) and $\times 20$ (**d**). M-OS cells at passage 30 (A30) with 50 % confluence, presence of a

cell cluster, $\times 10$ (**b**) and $\times 20$ (**e**). M-OS cells at passage 50 (A50) with 50 % confluence, presence of larger cells with cytoplasmic extensions, $\times 10$ (**c**) and $\times 20$ (**f**)

Table 1 Distribution of chromosome number in 100 meta-phase spreads of the M-OS cell line

	M-OS
Chromosome number, range	24–96
Near-Haploid (23–34), %	4
Near-Diploid (35–57), %	55
Near-Triploid (58–80), %	10
Near-Tetraploid (81–96), %	31

COX2 and the mesenchymal marker VIMENTIN. The proliferation marker KI67 was quantified and found in 90 % of the 10th passage cells and in 50 % of the 30th and 50th passage cells. The positivity for P53 was taken when more than 10 % of cells presented mutant P53, which was detected in all passages of M-OS (Fig. 1 Supplementary Material).

Discussion

It is known that OS lung metastases have different biological properties from the primary tumours

(Krishnan et al. 2005). Therefore, treatments targeting OS primary tumours are not effective in treating the metastatic disease. In this sense, the better understanding of the OS metastatic behavior could be useful to provide molecular targets for new therapeutic regimens (PosthumaDeBoer et al. 2011). The majority of OS metastatic cell lines described are generated in vitro from OS primary tumours, usually in murine models (Gillette et al. 2008; Khanna et al. 2000; Kimura et al. 2002; Chen et al. 2009), but not directly from specimens of lung metastases. To the best of our knowledge, this is the first report about the establishment of a new human OS cell line M-OS generated from a nodule of OS lung metastasis, which was characterized by conventional cytogenetics, FISH and immunohistochemical analysis.

The M-OS cell line is currently in the 55th passage and presents spindle-shaped cell morphology and disorganized cell growth pattern probably due to loss of contact inhibition. Normal somatic cells can replicate only a limited number of times before they reach senescence, with the maximum number of replicative cycles of 50 population doublings in cell culture, often being referred as the ‘Hayflick limit’ (Reddel 2000).

Fig. 2 Karyogram of a GTG-banded metaphase spread from the M-OS cell line at passage 50, a near-tetraploid karyotype 91, XXYY, -14

Table 2 Quantity of staining cells by immunohistochemistry^a

Antibody	M-OS 10th passage		M-OS 30th passage		M-OS 50th passage	
	Positive N (%)	Negative N (%)	Positive N (%)	Negative N (%)	Positive N (%)	Negative N (%)
OSTERIX	100 (100.0)	0 (0.0)	90 (90.0)	10 (10.0)	90 (90.0)	10 (10.0)
OSTEOCALCIN	100 (100.0)	0 (0.0)	100 (100.0)	0 (0.0)	100 (100.0)	0 (0.0)
VIMENTIN	100 (100.0)	0 (0.0)	80 (80.0)	20 (20.0)	80 (80.0)	20 (20.0)
COX2	100 (100.0)	0 (0.0)	80 (80.0)	20 (20.0)	80 (80.0)	20 (20.0)
BCL2	100 (100.0)	0 (0.0)	80 (80.0)	20 (20.0)	80 (80.0)	20 (20.0)
KI67	90 (90.0)	10 (10.0)	50 (50.0)	50 (50.0)	50 (50.0)	50 (50.0)
P53	20 (20.0)	80 (80.0)	20 (20.0)	80 (80.0)	25 (25.0)	75 (75.0)

^a Evaluation of 100 cells for each passage of M-OS

Cytogenetic findings revealed that most OS specimens present complex chromosomal abnormalities (Biegel et al. 1989). The most common numerical

chromosomal abnormalities found in OS were +1, -3, -9, -10, -13, -15 and -17, but multiple clones with different ploidy levels were also reported (Bridge et al.

1997; Mertens et al. 1993). In this study, the most common numerical alterations in the M-OS cell line were the losses of chromosomes 9, 13, 14, 16, 17, 19, 21, 22 and Y. Also, we detected in M-OS a high cell-to-cell heterogeneity regarding the chromosome number and two cell populations with different ploidy levels could be separated, one near-diploid and other near-tetraploid. Chromosomal instabilities characterized by heterogeneous divisions as bipolar and multipolar mitoses are responsible for the large number of nonclonal cells, presenting chromosome numbers with high cell-to-cell variability (Jin et al. 2007).

The FISH analysis of *RB1* and *MDM2* genes revealed that the copy number gain of *MDM2* without amplification was the most frequent alteration, while the homozygous deletion of *RB1* was detected only in a small number of cells. Indeed, *MDM2* copy number gain without amplification has already been reported as a frequent event of conventional OS subtypes and a rare event in parosteal OS, that is characterized by *MDM2* amplification (Duhamel et al. 2012).

The immunohistochemical analysis confirmed that M-OS cell line maintained the osteoblast-specific markers (OSTERIX and OSTEONALCIN) and the mesenchymal marker (VIMENTIN) even after all passages for the cell line establishment in vitro. These osteogenic and mesenchymal markers were equally expressed in the 10th, 30th and 50th passages of the cell line. We could also detect in M-OS the expression of the anti-apoptotic protein BCL2, the inflammation modulator COX2 and the mutant P53 protein.

In summary, we described for the first time the establishment of M-OS, a human OS cell line derived directly from a pulmonary metastasis nodule of OS. The cytogenetic characterization of M-OS was marked by a large number of nonclonal cells with high heterogeneity regarding the chromosome number, with different ploidy levels ranging from near-diploid to near-tetraploid. Moreover, the osteogenic nature and mesenchymal phenotype of M-OS were confirmed by immunohistochemical analysis. Taken together, these findings provide preliminary evidences to the new OS cell line, which could be used in the future in functional assays of cell culture and manipulated biological systems in order to characterize its tumorigenicity potential and better understand the mechanisms of invasion and metastatic establishment of OS cells.

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References

- Bacci G, Rocca M, Salone M, Balladelli A, Ferrari S, Palmerini E, Forni C, Briccoli A (2008) High grade osteosarcoma of the extremities with lung metastases at presentation: treatment with neoadjuvant chemotherapy and simultaneous resection of primary and metastatic lesions. *J Surg Oncol* 98:415–420. doi:[10.1002/jso.21140](https://doi.org/10.1002/jso.21140)
- Biegel JA, Womer RB, Emanuel BS (1989) Complex karyotypes in a series of pediatric osteosarcomas. *Cancer Genet Cytogenet* 38:89–100. doi:[10.1016/0165-4608\(89\)90169-6](https://doi.org/10.1016/0165-4608(89)90169-6)
- Bridge JA, Nelson M, McComb E, McGuire MH, Rosenthal H, Vergara G, Maale GE, Spanier S, Neff JR (1997) Cytogenetic findings in 73 osteosarcoma specimens and a review of the literature. *Cancer Genet Cytogenet* 95:74–87. doi:[10.1016/S0165-4608\(96\)00306-8](https://doi.org/10.1016/S0165-4608(96)00306-8)
- Chen X, Yang TT, Wang W, Sun HH, Ma BA, Li CX, Ma Q, Yu Z, Fan QY (2009) Establishment and characterization of human osteosarcoma cell lines with different pulmonary metastatic potentials. *Cytotechnology* 61:37–44. doi:[10.1007/s10616-009-9239-3](https://doi.org/10.1007/s10616-009-9239-3)
- Duhamel LA, Ye H, Halai D, Idowu BD, Presneau N, Tirabosco R, Flanagan AM (2012) Frequency of mouse double minute 2 (MDM2) and mouse double minute 4 (MDM4) amplification in parosteal and conventional osteosarcoma subtypes. *Histopathology* 60:357–359. doi:[10.1111/j.1365-2559.2011.04023.x](https://doi.org/10.1111/j.1365-2559.2011.04023.x)
- Gillette JM, Gibbs CP, Nielsen-Preiss SM (2008) Establishment and characterization of OS 99–1, a cell line derived from a highly aggressive primary human osteosarcoma. *In Vitro Cell Dev Biol Anim* 44:87–95. doi:[10.1007/s11626-007-9075-8](https://doi.org/10.1007/s11626-007-9075-8)
- Honoki K, Mori T, Tsutsumi M, Tsujiuchi T, Kido A, Morishita T, Miyauchi Y, Dohi Y, Mii Y, Tamai S, Konishi Y (1998) Heterogeneous pattern of gene expression in cloned cell lines established from a rat transplantable osteosarcoma lung metastatic nodule. *Cancer Lett* 127:221–228. doi:[10.1016/S0304-3835\(98\)00048-2](https://doi.org/10.1016/S0304-3835(98)00048-2)
- Jin Y, Stewenius Y, Lindgren D, Frigyesi A, Calcagnile O, Jonson T, Edqvist A, Larsson N, Lundberg LM, Chebil G, Liedberg F, Gudjonsson S, Månsson W, Höglund M, Gisselsson D (2007) Distinct mitotic segregation errors mediate chromosomal instability in aggressive urothelial cancers. *Clin Cancer Res* 13:1703–1712. doi:[10.1158/1078-0432.CCR-06-2705](https://doi.org/10.1158/1078-0432.CCR-06-2705)
- Khanna C, Prehn J, Yeung C, Caylor J, Tsokos M, Helman L (2000) An orthotopic model of murine osteosarcoma with clonally related variants differing in pulmonary metastatic potential. *Clin Exp Metastasis* 18:261–271. doi:[10.1023/A:1006767007547](https://doi.org/10.1023/A:1006767007547)
- Kimura K, Nakano T, Park YB, Tani M, Tsuda H, Beppu Y, Moriya H, Yokota J (2002) Establishment of human

- osteosarcoma cell lines with high metastatic potential to lungs and their utilities for therapeutic studies on metastatic osteosarcoma. *Clin Exp Metastasis* 19:477–485. doi:[10.1023/A:1020395816633](https://doi.org/10.1023/A:1020395816633)
- Krishnan K, Khanna C, Helman LJ (2005) The biology of metastases in pediatric sarcomas. *Cancer J* 11:306–313. doi:[10.1097/00130404-200507000-00006](https://doi.org/10.1097/00130404-200507000-00006)
- Mertens F, Mandahl N, Orndal C, Baldetorp B, Bauer HC, Rydholm A, Wiebe T, Willén H, Åkerman M, Heim S, Mittelman F (1993) Cytogenetic findings in 33 osteosarcomas. *Int J Cancer* 55:44–50. doi:[10.1002/ijc.2910550109](https://doi.org/10.1002/ijc.2910550109)
- Mirabello L, Troisi RJ, Savage SA (2009) Osteosarcoma incidence and survival rates from 1973 to 2004: data from the surveillance, epidemiology, and end results program. *Cancer* 115:1531–1543. doi:[10.1002/cncr.24121](https://doi.org/10.1002/cncr.24121)
- Petrilli AS, de Camargo B, Filho VO, Bruniera P, Brunetto AL, Jesus-Garcia R, Camargo OP, Pena W, Péricles P, Davi A, Prospero JD, Alves MT, Oliveira CR, Macedo CR, Mendes WL, Almeida MT, Borsato ML, dos Santos TM, Ortega J, Consentino E; Brazilian Osteosarcoma Treatment Group Studies III and IV (2006) Results of the brazilian osteosarcoma treatment group (BOTG) studies III And IV: prognostic factors and impact on survival. *J Clin Oncol* 24:1161–1168. doi:[10.1200/JCO.2005.03.5352](https://doi.org/10.1200/JCO.2005.03.5352)
- PosthumaDeBoer J, Witlox MA, Kaspers GJ, van Royen BJ (2011) Molecular alterations as target for therapy in metastatic osteosarcoma: a review of literature. *Clin Exp Metastasis* 28:493–503. doi:[10.1007/s10585-011-9384-x](https://doi.org/10.1007/s10585-011-9384-x)
- Reddel RR (2000) The role of senescence and immortalization in carcinogenesis. *Carcinogenesis* 21:477e84
- Sandberg M, Bridge JA (1994) The cytogenetics of bone and soft tissue tumors. RG Landes Co, Austin
- Shaffer LG, Tommerup N (2009) ISCN. An international system for human cytogenetic Nomenclature. S Karger, Basel