Establishment of a new human osteosarcoma cell line, UTOS-1: cytogenetic characterization by array comparative genomic hybridization

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Abstract

The cytogenetic characteristics of osteosarcoma (OS) remain controversial. The establishment of a new human OS cell line may improve the characterization. We report the establishment of a new human osteosarcoma cell line, UTOS-1, from a typical osteoblastic OS of an 18-year-old man. Cultured UTOS-1 cells are spindle-shaped, and have been maintained in vitro for over 50 passages in more than 2 years. Xenografted UTOS-1 cells exhibit features typical of OS, such as production of osteoid or immature bone matrix, and proliferation potency in vivo. UTOS-1 also exhibit morphological and immunohistochemical characteristics typical of osteoblastic OS. Chromosomal analysis by G-band show 73~85 chromosomes with complicated translocations. Array CGH show frequent gains at locus DAB2 at chromosome 5q13, CCND2 at 12p13, MDM2 at 12q14.3-q15, FLI and TOP3A at 17p11.2-p12 and OCRL1 at Xq25, and show frequent losses at HTR1B at 6q13, D6S268 at 6q16.3-q21, SHGC17327 at 18pter, and STK6 at 20q13.2-q13.3. The UTOS-1 cell line may prove useful for biologic and molecular pathogenetic investigations of human OS.

Introduction

Osteosarcoma (OS) is the most common malignant bone tumor in adolescents and young adults, and is characterized by proliferation of tumor cells which produce osteoid or immature bone matrix. Despite recent advances in multimodality treatment consisting of aggressive adjuvant chemotherapy and wide local excision, pulmonary metastasis occurs in approximately 40 to 50% of patients with OS and remains a major cause of fatal outcome [1-3].

There have been several reports describing xenotransplantation models of human OS [4-7], but characterization of human OS at the molecular cytogenetic level has been limited [8,9]. We describe the establishment and characterization of a new human OS cell line, designated as UTOS-1, derived from a conventional osteoblastic OS. In addition, we analyze chromosomal aberrations and DNA copy number changes in UTOS-1 by array comparative genomic hybridization (aCGH).
Methods
Source of Tumor Cells
An 18-year-old Japanese man noticed swelling and pain of the left shoulder for 2 months. Radiographs showed a periosteal reaction and an osteosclerotic change in the proximal metaphysis of the left humerus. An open biopsy of this humeral tumor confirmed that it was conventional osteoblastic OS (Figure 1). Immunohistochemically, most of the tumor cells were strongly positive for vimentin, alkaline phosphatase (ALP), osteopontin (OP), and osteocalcin (OC). Despite intensive chemotherapy, the patient died of lung metastasis 2 months after open biopsy. The present study was conducted after a human experimentation review by our ethics committee.

Tumorigenicity in severe combined immunodeficiency (SCID) mice
To determine the tumorigenicity of the UTOS-1 cell line in vivo, 1 × 10^8 cells were washed, suspended in phosphate-buffered saline (PBS), and injected subcutaneously into the leg of 4-week-old male SCID mice (CB-17/1scid; Clea Japan Incorporation, Osaka, Japan). Also, tumor growth in vivo was measured by calculating tumor volume based on the measurement of 2 perpendicular diameters using a caliper [10]. The volume was estimated using the following formula: 0.5 × L × (S)^2, where L and S are the largest and smallest perpendicular tumor diameters, respectively.

Establishment of the tumor cell line
Tumor cells were seeded in a 25 cm^2 plastic flask (35–3109; Falcon, Franklin Lakes, NJ, USA) [11]. These cells were cultured in RPMI 1640 (MP Biomedicals, Solon, OH, USA), supplemented with 100 mg/ml streptomycin (Meiji Seika, Tokyo, Japan), 100 U/ml penicillin (Meiji Seika) and 10% fetal bovine serum (FBS; Funakoshi, Tokyo, Japan), at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The medium was replaced once per week. When semiconfluent layers were obtained, the cells were dispersed with Ca^{2+}- and Mg^{2+}-free PBS containing 0.1% trypsin and 0.02% EDTA solution, and were then seeded in new flasks for passage. The configuration of tumor cells was almost equalized after the 3rd generation. These procedures were serially performed until the UTOS-1 cell line was established.

Cell growth in vitro
To determine the doubling time, UTOS-1 cells were seeded in each well of 96-well dishes (Corning Costar, Tokyo, Japan) with fresh medium containing 100 μl of RPMI 1640 with 10% FBS. Cell growth was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Cell Counting Kit-8, Dojindo, Tokyo, Japan) [12]. A volume of 10 μl of MTT was added to each well, followed by mixing. Plates were incubated for 3 hours at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Formazan levels, which correspond to the number of viable cells, were quantified using a microplate reader (model 450; Bio-Rad Laboratories, Hercules, CA, USA) at a wavelength of 450 nm. The absorbance of each well was evaluated at 6, 12, 24, 48, 72, 96 and 120 hours after seeding. Triplicate wells were used for each observation.

Immunohistochemistry
Cells were cultured in chamber slides (Lab-Tek; Nalge Nunc International, Naperville, IL, USA). For the detection of mesenchymal phenotype, we used 3 monoclonal antibodies: anti-AE1/AE3, anti-keratin mix, and anti-vimentin. Also, to assess osteoblastic differentiation, we used 2 monoclonal antibodies: anti-OP and anti-OC. ALP activity of UTOS-1 cells was estimated using a modified version of a cytochemical method described elsewhere [13], with naphthol AS-MX phosphate-fast blue RR staining (ALP staining kit; Muto Pure Chemicals Corporation, Tokyo, Japan).

RNA extraction and reverse-transcription polymerase chain reaction (RT-PCR)
Expression of osteoblastic differentiation markers was assessed using RT-PCR. UTOS-1 cells were grown to con-
fluence, and total cellular RNA was isolated using a TRI-
zol® Reagent (Invitrogen, San Diego, CA, USA). Total RNA
was used as a template for cDNA synthesis using the
SuperScript First-strand Synthesis System (Invitrogen).
PCR was performed to assess expression of ALP, OP, and 
OC. The oligonucleotide primer sequences and PCR con-
ditions for ALP, OP and OC are shown in Table 1. Ampli-
fied products were analyzed by 2% agarose gel (Cambrex
Bio Science Rockland Incorporation, Rockland, ME, USA)
electrophoresis and ethidium bromide staining (Invitro-
gen). For comparison, Saos-2 [7], which is one of the most
popular OS cell lines, was used as a positive control.

Cytogenetic analysis
For cytogenetic analysis, preparations of metaphase chro-
mosomes from UTOS-1 cells at passage 15 were obtained,
and were banded with Giemsa-trypsin [14]. Karyotypes
were described using the short version of the International
System for Human Cytogenetic Nomenclature [15].

DNA extraction and array CGH
Genomic DNA was extracted from UTOS-1 cells at passage
15. The CGH procedure used was similar to published
standard protocols [16]. Genomic DNA was isolated from
tumor samples using standard procedures including pro-
teinase K digestion and phenol-chloroform extraction.

Array CGH was performed using the GenoSensor Array
300 system, following the manufacturer's instructions
(Vysis, Downers Grove, IL, USA). This array contains the
287 chromosomal regions that are commonly altered in
human cancer, such as telomeres, regions involved in
microdeletions, oncogenes, and tumor suppressor genes.
Tumor DNA (100 ng) was labeled by random priming
with fluorolink cy3-dUTP, and normal reference (control)
DNA was labeled using the same method with cy5-dUTP.
The tumor and control DNAs were then mixed with Cot-
1 DNA (GIBCO-BRL, Gaithersburg, MD, USA), precipi-
tated, and resuspended in microarray hybridization buffer
containing 50% formamide. The hybridization solution
was heated to 80°C for 10 minutes to denature the DNA,
and was then incubated for 1 hour at 37°C. Hybridization
was performed for 72 hours in a moist chamber, followed
by a post-hybridization wash in 50% formamide/2 × SCC
at 45°C. Slides were mounted in phosphate buffer con-
taining 4',6-diamidino-2-phenylindole (DAPI; Sigma, St.
Louis, MO, USA). Fluorescence intensity images were
obtained using the GenoSensor Reader System (Vysis)
according to the manufacturer's instructions. For each
spot, the total intensity of each of the 2 dyes and the ratio
of their intensities were automatically calculated. The
diagnostic cut-off levels representing gains and losses were
set at 1.2 (upper threshold) and 0.8 (lower threshold).
This assay was performed in triplicate, and common aber-
rations were considered to be meaningful aberrations.

Results
Tumor growth in vivo
Approximately 5 weeks after implantation, all SCID mice
had palpable elastic hard nodules with a volume of about
1000 mm³ (Figure 2). The tumor volume was about 4000
mm³ at 6 weeks after implantation, and was > 10,000
mm³ at 8 weeks after implantation. The cut surfaces of
these tumors were solid and white-gray with small
necrotic foci. Histopathologically, the tumors contained
primarily atypical tumor cells, and exhibited formation of
osteoid or immature bone matrix, which is similar in
characteristics to the original tumor (Figure 3).

Cell growth and morphological findings in vitro
UTOS-1 cells were spindle-shaped, contained several
nucleoli, and formed clumps. Two weeks after initial cul-
tivation in primary culture, the tumor cells reached sub-
confluence with some piled-up foci of cells (Figure 4A).
After the cells were serially subcultured for about 3
months, they began to grow rapidly at passage 6 (Figure
4B).

This new cell line has been maintained in vitro for more
than 50 passages over more than 2 years. In the exponen-
tial phase of cell growth, the population-doubling time
was 40 hours (Figure 5).

Table 1: The oligonucleotide primer sequences and PCR conditions for ALP, OP, and OC in this study.

| Molecule | Primers (5’ to 3’) | Strand | Size (bp) | Conditions (temperature, cycle number) |
|----------|--------------------|--------|-----------|---------------------------------------|
| ALP      | ACGTGGCTAAGAATGTCATC CTGGTAGGCGATGTCCTTA | +      | 475       | 55°C 35 cycles                       |
|          |                    | -      |           |                                       |
| OP       | CCAAGTAAGTCCCAAAGAAG GTGATGTCCTCGTCTGTA | +      | 347       | 58°C 45 cycles                       |
|          |                    | -      |           |                                       |
| OC       | ATGAGGCCCTCACACTCCTC GCCGTAGAAGCGCGGATAGGC | +      | 294       | 59°C 45 cycles                       |
|          |                    | -      |           |                                       |
| GAPDH    | GAAGGTGAGTGTCGGGATGCA GAGATGGTGTAGGGATTTTC | +      | 226       | 55°C 35 cycles                       |

Abbreviations: ALP, alkaline phosphatase; OP, osteopontin; OC, osteocalcin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Immunohistochemical and cytochemical findings

All UTOS-1 cells were negative for AE1/AE3 and keratin mix. Most UTOS-1 cells were positive for vimentin. All UTOS-1 cells were positive for OP, OC and ALP (Figure 6).

RT-PCR

UTOS-1 cells expressed ALP, OP and OC, which is similar to the results for Saos-2 (Figure 7).

Cytogenetic findings

A representative karyotype is shown in Figure 8. 50 UTOS-1 cells exhibited a complex karyotype. The karyotypes of UTOS-1 cells at passage 15 were similar to those of the original tumor. The composite karyotypes were as follows:

73-85, Y, -X [7]+Y[10],add(X)(q11)[9],add(X)(q11)[2],+1[8],del(1)(q11)[9],der(1)add(1)(p11)add(1)(q42)[9],der(1)add(1)(p22)add(1)(q32)[2],der(1)add(1)(p32)add(1)(q42)[6],-3[10],-4[3],add(4)(q11)[9],-5[4],del(5)(p13)[9],add(6)(q11)[3],der(6)del(6)(p21)add(6)(q22)[10],der(6)del(6)(p24)add(6)(q13)×2,[10]-7[10],add(7)(p22)[6],der(7)(7;7)[p22;q22][10],+8[3],-9[10],-9[8],add(9)(q22)[9],-10[10],add(10)(p11)[4],add(10)(q26)[7],der(10)add(10)(p11)add(10)(q26)[3],add(11)(p11)[9],add(11)(p11)[4],del(11)(p11)[6],-12[5],der(12)(q21)[6],der(12)add(12)(p11)add(12)(q24)[10],der(12)add(12)add(12)[7],-13[10],+14[2],add(14)(p11)[10],add(14)[2],add(14)(p11)[8],-15[7],add(15)(p11)[5],add(15)(p11)[4]+16[5],add(16)(p11)[3],

Figure 2

Tumor volume in SCID mice. Tumor volume in logarithmic growth phase, ~5 weeks after inoculation. Values are expressed as the mean ± standard deviation of triplicate cultures.

Figure 3

Histologic appearance of xenografted tumor in SCID mice. A. Xenografted tumor showing features typical of osteoblastic osteosarcoma with atypical spindle-shaped cells (H&E stain). Scale bar: 100 μm. B. The proliferation of atypical tumor cells with osteoid formation is shown. Xenografted tumor cells resemble original tumor cells. Scale bar: 50 μm.

Figure 4

Morphology under phase-contrast microscopy. A. In primary culture, spindle-shaped tumor cells reach subconfluence with some piled-up foci of cells. Scale bar: 100 μm. B. At passage 6, the tumor cells begin to grow rapidly. The configuration of tumor cells is equalized after the 6th generation. Scale bar: 100 μm.

Figure 5

Tumor cell growth in vitro. UTOS-1 cells begin to grow ~24 hours after inoculation. The population-doubling time of the cells is 40 hours. Values are expressed as the mean ± standard deviation of triplicate cultures.
add(16)(q24)[2], add(16)(p11)[10], add(16)(p11)[4], -17[10], -17[8], add(17)(q24)[3], del(17)(p11)[3]. -18[5], add(18)(p11)[9], add(18)(q21)[5], +19[5], add(19)(p11)[9], add(19)(q13)[8], del(19)(p13)[9], +20[7], add(20)(p11)[7], add(20)(p13)[8], add(20)(q11)[4], +21[8], +21[4], add(21)(p11)[10], add(21)(p11)[4], add(21)(q22)[7], +22[10], +22[7], +22[3], del(22)(q13)[10], del(22)(q9)[10], +10~18 mar.

Array CGH

Significant gains of DNA sequences were observed for locus DAB2 at chromosome 5q13, CCND2 at 12p13, MDM2 at 12q14.3-q15, FLI, TOP3A at 17p11.2-p12, and OCRL1 at Xq25. Significant losses of DNA sequences were observed for HTR1B at 6q13, D6S268 at 6q16.3-q21, SHGC17327 at 18pter, and STK6 at 20q13.2-q13.3. The representative aCGH profile is shown in Figure 9.

Discussion

There have been several reports describing xenotransplantation models of human OS [4-7]. In the present study, the parent tumor, the cultured tumor cells, and the xenografted tumor exhibited features typical of OS, as

Figure 6
Immunohistochemical findings. A, B. UTOS-1 cells are negative for AE1/AE3 and keratin mix. C, D, E. Most UTOS-1 cells are positive for vimentin, OP, and OC. F. Staining for ALP was performed using a modified cytochemical method. ALP activity is visible as blue staining. UTOS-1 cells are strongly positive for ALP.

Figure 7
Osteoblast marker expression in UTOS-1 cells. The expression of several osteoblast markers, including ALP, OP and OC, is shown. Saos-2, which is one of the most popular OS cell lines, is used as a positive control for osteoblastic markers in UTOS-1 cells. These cells express ALP, OP and OC, which is similar to Saos-2.

Figure 8
A representative G-banded karyotype of a UTOS-1 cell. Arrows show the abnormal chromosomes.
reported previously [15,17]. Cultured UTOS-1 cells have a spindle shape with several nucleoli, which is similar to the original tumor cells. Biochemical characteristics of UTOS-1, such as cell growth rate and osteoblastic activity, have not changed during the 2 years that they have been maintained. Immunohistochemically, the UTOS-1 cells remain positive for ALP, OP and OC. After implantation from cell cultures into SCID mice, UTOS-1 cells grew in vivo, producing osteoid resembling that of the original tumor. Abundant osteoid tissue formed in the xenografted tumors and reimplanted tumors. These findings suggest that UTOS-1 cells have an osteoblastic phenotype and retain the characteristics of the original tumor. The population-doubling time of UTOS-1 cells in vitro is 40 hours, which is similar to that of other OS cell lines [4,6,18].

Several reports indicate that OS cells have karyotypes with multiple numerical rearrangements and complex structural rearrangements [9,19-21]. Together, the results of several cytogenetic surveys indicate that OS cells frequently have structural alterations at chromosome bands 1p11-13, 1q11-12, 1q21-22, 11p15, 12p13, 17p11-3, 19q1, and 22q11-13, and frequently have the numerical chromosome abnormalities +1, -9, -10, -13, and -17. In UTOS-1 cells, the clonal chromosomal abnormalities that were detected were triploidies. The chromosomal rearrangements that were observed in UTOS-1 involved chromosomes 1q11-12, 11p15, 19q13, and 22q11-13. The numerical chromosome abnormalities that were observed in UTOS-1 included +1, -9, -10, -13, and -17. These findings are similar to studies of other OS cell lines [8].

Metaphase CGH studies of OS have identified frequent gains at chromosome bands 1p32, 1q21, 5p13, 6p12, 8q24, 8cen-q13, 17p11.2, and Xp21, and frequent losses at bands 6q16, 10p12pter, and 10q22-q26 [22,23]. Recent metaphase CGH studies of OS have focused on amplifications at chromosomes 8q, 6p, and 17p [22,24]. Advances in mapping resolution of microarray CGH [25,26] have greatly improved its resolving power, such that it now provides greater detail than metaphase CGH regarding the complexity and exact location of genomic rearrangements leading to copy number imbalances.

In the present study, chromosome 12 showed several distinct regions of focal amplification, occurring at gains of CCND2 at 12p13 12q13 and MDM2 at 12q14.3-q15. Previous CGH studies of OS have revealed abnormalities of chromosome 12, including gains at bands 12p12-p13 [24], 12q12-q13 [27], and 12q13-q14 [28]. Expression of the CCND2 gene, which is located at chromosome 12p13, has been observed in various malignancies, including prostate cancer and breast cancer [29-31]. CCND2 encodes a protein belonging to the cyclin family of proteins that regulate cyclin-dependent kinase (CDK) kinases [32]. CDK activity controls the cell cycle G1/S transition by regulating phosphorylation of the tumor suppressor protein Rb [33]. These facts suggest that CCND2 controls proliferation of UTOS-1 tumor cells.

Some studies indicate that 14 to 27% of OS tumors have abnormal MDM2 expression [34,35]. MDM2 is a target gene of the transcription factor tumor protein p53 [36]. The encoded protein is a nuclear phosphoprotein that binds and inhibits transactivation by tumor protein p53, as part of an autoregulatory negative feedback loop [37,38]. Overexpression of MDM2 gene can result in excessive inactivation of tumor protein p53, diminishing its tumor suppressor function. These findings suggest the possible involvement of the p53 tumor suppressor gene, which is associated with development of OS in UTOS-1 cells.

The gain of chromosome band at 17p11.2-p12 has been observed in approximately 13 to 29% of high-grade OS tumors [24,39,40]. In UTOS-1 cells, gain of the genes FLI and TOP3A at chromosome 17p11.2-p12 has been observed. These findings suggest that multiple gains, including FLI, TOP3 or other genes close to these candidate oncogenes, are present at chromosome 17p11.2-p12 and contribute to OS tumorigenesis [41]. Recent studies indicate that overexpression of 17p11.2-p12 is associated with p53 degradation [42-44].

In a study of OS using a cDNA array, Squire et al. observed amplification of the genes MYC, GAS7, and PM1 in OS cells [45]. Other reports indicate that losses of chromosome bands 6q16 and 6q21-q22 occur in high-grade OS [46]. These findings and those of the present study suggest that gene losses on chromosome 6q, including HTR1B and D6S268, contribute to OS tumorigenesis.

One of the most remarkable breakpoint clusters that have been found in OS tumors was detected on chromosome...
20 by spectral karyotyping (SKY) analysis [47]. Chromosome 20 is one of the smaller chromosomes, suggesting that it is particularly vulnerable to structural rearrangement. However, there is little evidence that chromosome 20 is frequently involved in chromosomal imbalances [26,28]. In the present study, the only loss that involved chromosome 20 occurred at band 20q13.2-q13.3. Many chromosomal changes have been observed in CGH studies of high-grade OS [46]. Reports indicate that the genes involved in OS tumorigenesis include DAB2 (at chromosome 5q13), OCR1L1 (at Xq25), and SHGC17327 (at 18pter). However, many of these genes were not previously known to be associated with OS tumorigenesis.

In conclusion, we have isolated and characterized a new permanent human cell line, UTOS-1, established from an osteoblastic OS. This cell line retains the morphology, osteoblastic activities and cytogenetic characteristics of the original tumor in vitro. The UTOS-1 cell line is useful for biologic and molecular pathogenetic studies of human OS.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
Authors have made substantial contributions to conception and design MK and TY acquisition of data. SN, TH, KS analysis, interpretation of data, organizing study. TY and supervision of research group TK

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