Analysis of dihydrofolate reductase and reduced folate carrier gene status in relation to methotrexate resistance in osteosarcoma cells

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Abstract

Background: To evaluate the impact of dihydrofolate reductase (DHFR) and reduced folate carrier (RFC) genes on methotrexate (MTX) resistance in osteosarcoma cells in relation to retinoblastoma (RB1) gene status.

Materials and methods: A series of human osteosarcoma cell lines—either sensitive or resistant to MTX—and 16 osteosarcoma tumour samples were used in this study.

Results: In U-2OS MTX-resistant variants, and in other RB1-positive cell lines, MTX resistance was associated with increased levels of DHFR and with a slight decrease of RFC gene expression. In Saos-2 MTX-resistant variants, and in another RB1-negative cell line, development of MTX resistance was associated with a decrease in expression of RFC, without any significant involvement of DHFR. In osteosarcoma clinical samples, amplification of the DHFR gene at clinical onset appeared to be more frequent in RB1-positive compared with RB1-negative tumours.

Conclusions: Amplification of the DHFR gene may occur more frequently in the presence of RB1-mediated negative regulation of its activity and can be present at clinical onset in osteosarcoma patients. Simultaneous evaluation of RFC, DHFR and RB1 gene status at the time of diagnosis may become the basis for the identification of potentially MTX-unresponsive osteosarcoma patients, who could benefit from treatment protocols with alternative antifolate drugs.

Key words: dihydrofolate reductase, drug resistance, methotrexate, osteosarcoma, reduced folate carrier, retinoblastoma gene

Introduction

Osteosarcoma, the most common primary malignant tumour of bone, is characterised by an extremely aggressive clinical course with rapid development of metastases in 40–50% of patients [1–3]. Although the adoption of adjuvant and neoadjuvant chemotherapy has significantly improved the prognosis of this tumour, a considerable number of osteosarcoma patients develop drug resistance and die as a result of disease progression [4–6].

Methotrexate (MTX) is a common constituent of chemotherapeutic regimens for high-grade osteosarcoma, together with doxorubicin, cisplatin and ifosfamide [7, 8]. MTX is a potent inhibitor of the dihydrofolate reductase (DHFR) enzyme which plays a key role in intracellular folate metabolism and is essential for DNA synthesis and cell growth [9, 10]. Studies performed on experimental models, as well as on different human cancers, have shown that resistance to MTX can arise through different mechanisms, which vary from tumour to tumour [9, 11]. Although MTX is one of the most important drugs in osteosarcoma treatment, only one report has been published so far about the mechanisms through which this tumour may become clinically resistant to the drug [12]. These mechanisms include the increase of DHFR enzyme levels (due to DHFR gene overexpression) and the impaired intracellular transport of MTX as a consequence of decreased levels of reduced folate carrier (RFC) at the cell membrane [12].

Enhanced levels of DHFR are frequently present in proliferating tumour cells as a consequence of increased expression, which can be associated with amplification of the DHFR gene [13]. Expression of DHFR is negatively regulated by the retinoblastoma (RB1) gene [14–16], which therefore may play an important role in determining the sensitivity to drugs targeting DHFR [11]. The RB1 gene is a major regulator of cell proliferation and its deletion or mutation have been demonstrated to be responsible for loss of growth regulation in many tumour cells [14]. The RB1 gene regulates expression of genes which, like DHFR, encode for proteins required for DNA synthesis through interaction with the E2F family of transcriptional factors. Since the RB1 gene is frequently altered in osteosarcoma [17], the simultaneous analysis of RB1, DHFR and RFC gene status may contribute to a better understanding of their relative impact on MTX resistance in human osteosarcoma cells. This may also give important information for
the development of new therapeutic regimens with alternative antifolate drugs for high-grade osteosarcoma patients who are unresponsive to MTX. Many new antifolate compounds which have been designed to circumvent the known mechanisms of resistance to MTX are currently under clinical development and evaluation [11, 18–20]. Among these, trimetrexate has demonstrated antitumour activity against a number of malignant neoplasms; however, in advanced colorectal cancer the addition of trimetrexate to 5-fluorouracil/leucovorin did not increase overall survival [21]. Although trimetrexate activity has been analysed in several tumours [22], no data have been reported so far about its effectiveness in human osteosarcoma cells.

In this study, we have investigated the role of DHFR, RFC and RB1 genes in the development of MTX resistance in a series of MTX-resistant variants established from U-2OS (RB1-positive) or Saos-2 (RB1-negative) cell lines and in four additional human osteosarcoma cell lines. The status of the DHFR gene at clinical onset was also assessed in a series of either RB1-positive or RB1-negative high-grade osteosarcoma clinical samples, in order to determine whether it may assist in the identification of patients who are potentially unresponsive to MTX and could benefit from treatment protocols with alternative antifolate drugs.

Moreover, we assessed the in vitro effectiveness of trimetrexate in our cell lines in order to determine whether this antifolate drug may be considered for alternative chemotherapeutic regimens in osteosarcoma patients unresponsive to MTX.

Materials and methods

Chemicals

MTX, doxorubicin and cisplatin were purchased from Sigma-Aldrich (Milan, Italy). Trimetrexate (produced by U.S. BioPharma BV, Nijmegen, The Netherlands) was a generous gift of IPSEN S.p.A. (Milan, Italy). Stock solutions of MTX, doxorubicin and cisplatin were stored at 4°C. Trimetrexate was dissolved in distilled water and aliquots were stored at −20°C. For all the drugs, working concentrations were prepared by diluting stock solutions in culture medium immediately before use.

Cell lines

The human osteosarcoma cell lines U-2OS, Saos-2 and HOS were obtained from the American Type Culture Collection (ATCC; Rockville, MD). The human osteosarcoma cell lines SARG, IOR/OS18 and IOR/MOS were established at the Laboratorio di Ricerca Oncologica of the Istituti Ortopedici Rizzoli (Bologna, Italy) from clinical specimens obtained from untreated osteosarcoma patients. Starting from the MTX-sensitive U-2OS and Saos-2 human osteosarcoma cell lines, MTX-resistant variants were obtained by exposing parental cell lines to in vitro stepwise increased MTX concentrations. In vitro continuous exposure to MTX resulted in variants resistant to the following: 3 ng/ml (U-2OS/MTX3), 30 ng/ml (U-2OS/MTX30 and Saos-2/MTX30), 100 ng/ml (U-2OS/MTX100 and Saos-2/MTX100), 300 ng/ml (U-2OS/MTX300 and Saos-2/MTX300), or 1 µg/ml MTX (Saos-2/MTX1µg). These MTX concentrations correspond to 0.01 (3 ng/ml), 0.07 (30 ng/ml), 0.22 (100 ng/ml), 0.66 (300 ng/ml) and 2.20 µM MTX (1 µg/ml), respectively.

Parental cell lines were cultured in Iscove’s modified Dulbecco’s medium (IMDM), supplemented with penicillin (100 U/ml) and streptomycin (100 µg/ ml) (Gibco-BRL, Gaithersburg, MD, USA) and 10% fetal calf serum (FCS; Biological Industries, Kibbutz Beth Haemek, Israel). Each MTX-resistant variant was continuously cultured in the presence of the appropriate MTX concentration used for the selection. The HOS, SARG, IOR/OS18 and IOR/MOS cell lines were cultured as described for U-2OS and Saos-2. All cell lines were maintained at 37°C in a humidified 5% CO₂ atmosphere.

In vitro growth characteristics and chemosensitivity

To determine the in vitro growth rate of each cell line, 20 000 cells/cm² were seeded in IMDM 10% FCS in the absence of or with the appropriate concentration of MTX. Cells were then harvested and counted by Trypan blue dye exclusion every 24 h, and doubling time was calculated during the logarithmic phase of growth (from 48 to 96 h after seeding). For saturation density, from day 4 onwards, medium was changed daily and cells were counted every 2 days until they stopped growing. Cloning efficiency was determined by seeding between 200 and 50 000 cells in 60-mm dishes. Colonies were counted on day 14 after fixation with methanol and staining with Giemsa. The degree of MTX resistance was expressed as the ratio of the concentration resulting in 50% inhibition of growth (IC₅₀) of resistant variants to that of their corresponding parental cell lines. To determine the IC₅₀ values, 20 000 cells/cm² were seeded in IMDM 10% FCS and after 24 h the medium was replaced with IMDM 10% FCS and no MTX (control) or with different concentrations of MTX. After 96 h, cells were harvested and counted by Trypan blue dye exclusion to estimate the percentages of growth inhibition compared with the appropriate control, which were then used to calculate the IC₅₀ values. In vitro resistance to the other drugs was evaluated as described for MTX.

DNA and RNA extraction

Both DNA and RNA were isolated from frozen cell pellets (5–8 × 10⁶ cells) obtained for each cell line. High molecular weight DNA and total cellular RNA were isolated using DNAzol or TRIzol reagents (Gibco-BRL), respectively, according to the manufacturer’s instructions. After isolation, DNA and RNA concentrations were determined by spectrophotometry and the quality of DNA and RNA was evaluated by agarose–gel electrophoresis.

Quantitative real-time RT–PCR

Real-time fluorescence detection was used to analyse the relative levels of DHFR and RFC RNA. Total RNA (1 µg) was denatured at 65°C for 10 min and then reverse transcribed at 48°C for 40 min in a 100-µl reaction mixture containing 500 µM each dNTP, 125 U MultiScribe reverse transcriptase, 40 U Rnasin inhibitor, 2.5 µM oligo d(T)₃₀, 1× TaqMan RT buffer and 5 mM MgCl₂ (Applied Biosystems, Foster City, CA). Reactions omitting either the enzyme or the RNA were used as negative controls. In order to work under the same cycling conditions (95°C for 10 min, followed by 40 cycles at 95°C for 15 s, and 60°C for 1 min), TaqMan primers for the quantitative detection of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), human DHFR and human RFC were designed using Primer Express software (Applied Biosystems), which generated products with sizes of 224 (GAPDH) and 101 bp (DHFR and RFC). Primer sequences were as follows: GAPDH, forward, 5′-GAA GGT GAA GGT CGG AGT C-3′, reverse, 5′-GAA GAT GAT GAT GGG ATT TC-3′; DHFR, forward, 5′-ATG CCT TAA AAC TTA CTG AAC-3′, reverse, 5′-TGG GTG ATT CAT GGC TTC CT-3′; RFC, forward, 5′-TTG CTT TGC CAT CGT CCA AA-3′, reverse, 5′-ACA CGG AGT ACT GGA ACT TGC CT-3′. The relative standard curves for target and reference genes were made using 5-fold serial dilutions of U-2OS/MTX300 cDNA (3.2, 16, 80, 400, 2000, 10 000 pg/µl). All PCR reactions were performed in SYBRGreen PCR Master Mix 2× (containing SYBRGreen I dye, AmpliTaq Gold DNA polymerase, dNTP mix with dUTP, Passive Reference 1 and optimised buffer components) and ABI PRISM 7700 SDS instrument (Applied Biosystems). All samples were run in triplicate and were resolved on a 1.8% agarose gel to confirm PCR specificity of the amplifiers for DHFR and RFC.
For real-time data analysis, the mean concentration of GAPDH was used as a control for input RNA. GAPDH cDNA levels were determined for each cDNA sample and were used to normalise all other genes tested from the same cDNA sample. Relative amounts of DHFR and RFC cDNAs were calculated by comparison with standard curves. The relative RFC and DHFR mRNA expression was also normalised to the corresponding calibrator, consisting of U-2OS or Saos-2 mRNA. Final results were expressed as n-fold difference in RFC or DHFR expression relative to GAPDH and its corresponding calibrator.

Analysis of DHFR and RFC gene copy numbers

DHFR gene copy number was estimated by Southern blot and dot blot with a digoxigenin-labelled probe that was synthesised by PCR with the primers 5′-CAC TGT CTG ATT TCT GCC CGG ATT C-3′ and 5′-GAA GAG GTT GTG GTC ATT TCT TGG A-3′. Determination of the RFC gene copy number was performed by using a digoxigenin-labelled probe synthesised by PCR with the primers 5′-CAA AGC GCA GCC TCT TCT TCA ACC-3′ and 5′-CCA GCA CGG TGG AGG CAG CAT CTG CC-3′. For the Southern blots, 15 µg genomic DNA was digested with EcoRI overnight at 37°C (Roche Molecular Biochemicals, Mannheim, Germany) and then separated on a 0.8% agarose gel in TAE buffer. After denaturation and neutralisation, DNA samples were transferred onto positively charged, nylon membranes (Roche Molecular Biochemicals) and fixed by the membrane by UV cross-linking for 10 min followed by baking at 120°C for 30 min. For dot blot analysis, 10–20 µg target DNA was denatured by treatment with 0.4 M NaOH and 10 mM EDTA at 96°C for 10 min and then neutralised with 2 M ammonium acetate. Serial dilutions of each DNA sample were individually spotted onto positively charged nylon membranes (Roche Molecular Biochemicals) by using a 96-well Bio-Dot apparatus (BioRad, Richmond, CA), and then fixed to the membrane as described for Southern blotting. For both Southern blots and dot blots, membranes were pre-hybrised for 2–4 h at 68°C with standard hybridisation buffer (SHB; 0.02% lauryl sulfate, 5× SSC, 0.1% N-laurylsarcosin) supplemented with 1% blocking reagent (Roche Molecular Biochemicals). Blots were hybridised overnight at 68°C with 20 ng/ml probe, either DHFR or RFC, in SHB. Membranes were then washed at high stringency and the hybridisation signals were detected using an alkaline phosphatase chemiluminescence method (DIG Nucleic Acid Detection kit; Roche Molecular Biochemicals). After hybridisation with DHFR- or RFC-specific probes, membranes were stripped and then re-hybridised with a digoxigenin-labelled, human β-actin probe (Clontech, Palo Alto, CA) as a control of DNA loading. For each sample, DHFR or RFC gene copy numbers were determined by densitometric analysis in relation to the corresponding β-actin hybridisation signals.

Western blotting and immunofluorescence

For western blot analysis, cells were washed in cold PBS, collected by scraping and frozen at −80°C until protein extraction. Total protein extracts were prepared as described by Banerjee et al. [23] and protein concentration was determined by Lowry’s method. For each sample, 50 µg protein extract was mixed with Laemmli buffer, resolved on an SDS-polyacrylamide gel, and transferred to PVDF membranes (Roche Molecular Biochemicals). Western blot analyses were performed with anti-DHFR (clone 49; Becton Dickinson, Mountain View, CA, USA), anti-human pp110-114 retinoblastoma protein G3-245 (BD Pharmingen, San Diego, CA, USA), or anti-E2F-1 (clone KH95; Santa Cruz Biotechnology, Santa Cruz, CA, USA) monoclonal antibodies. Protein bands were visualised on X-ray film using an enhanced chemiluminescence detection system (ECL; Amersham, Little Chalfont, UK) according to the manufacturer’s protocols. For each sample, the amount of protein was determined by densitometric analysis.

The presence of RB1 protein (pRB) in each cell line was also assessed by indirect immunofluorescence with the monoclonal antibody G3-245 (BD Pharmingen).

Analysis of DHFR and RB1 gene status in osteosarcoma clinical samples

The analysis of DHFR gene copy number in the clinical samples was performed on DNA isolated from paraffin-embedded tumour tissue specimens. After dewaxing of between 5 and 10 representative 10 µm-thick sections of each case, tissue fragments were digested with 2 mg/ml type I collagenase (Sigma, Deisenhofen, Germany) followed by DNA isolation with DnaZol (Gibco-BRL). Good quality DNA, suitable for dot blot analysis, was isolated from 16 high-grade osteosarcomas, including 14 untreated primary tumours and two lung metastasis arisen after post-operative chemotherapy with high-dose MTX 12 g/m², doxorubicin 60–75 mg/m², cisplatin 120 mg/m² and ifosfamide 10 g/m². On the basis of the evaluation of tumour necrosis after pre-operative chemotherapy, a good histological response was considered when the extent of tumour necrosis was ≥90%.

For each sample, the DHFR gene copy number was determined by densitometric analysis in relation to the corresponding β-actin hybridisation signals. Amplification of the DHFR gene was defined in comparison with the signals obtained in the U-2OS cell line, which was used as an internal reference in each hybridisation experiment.

On the same cases, nuclear pRB was assessed by immunohistochemistry using the avidin–biotin–peroxidase method. After dewaxing, sections were incubated with citrate buffer solution (0.01 M citric acid, 0.01 M sodium citrate, pH 6.0) in a microwave oven at 750 W for three cycles of 5 min each in order to extract the antigen. Sections were incubated overnight at 4°C with the G3-245 monoclonal antibody (BD Pharmingen), and then with a biotinylated horse anti-mouse antibody and the avidin–biotin peroxidase complex (Vector Laboratories, Burkingame, CA, USA). The final reaction product was revealed by incubation with diaminobenzidine (Sigma) and nuclei were counterstained with Gill’s hematoxylin. Only tumour samples showing a positive nuclear immunostaining were classified as pRB positive.

Formalin-fixed, paraffin-embedded pellets of U-2OS and Saos-2 cell lines were used as positive and negative controls, respectively.

False-negative results were ruled out by adding, for each sample, a positive control for the antigenicity of the tumour specimen, which was incubated with the V9 anti-vimentin monoclonal antibody (Roche Molecular Biochemicals). Only samples that were negative for pRB but positive for vimentin were considered as pRB negative.

False-positive results were ruled out by adding, for each case, an additional section in which the primary antibody was replaced by non-specific mouse IgG. To consider the experiment as reliable, the result from this sample had to be completely negative.

Statistics

Two-tailed Fisher’s exact test was used to evaluate the statistical association between two variables.

Results

Selection and drug sensitivity of MTX-resistant variants

MTX-resistant variants of U-2OS and Saos-2 human osteosarcoma cell lines were obtained by initially exposing parental cell lines to 3 ng/ml (0.01 µM) MTX until they achieved a growth rate similar to that of untreated cells. Selection was then continued by stepwise increased MTX concentrations up to 300 ng/ml (0.66 µM) MTX for U-2OS variants, or to 1 µg/ml (2.20 µM) MTX for Saos-2 variants. Establishment of adequate in vitro growth at each new MTX concentration required approximately 8–12 weeks (corresponding to between six and 10 in vitro passages) for U-2OS cell lines and 6–8 weeks (corresponding to
between four and six in vitro passages) for Saos-2 cell lines. All the experiments were performed on cell lines maintained in culture for at least 6 months after selection with MTX.

The IC₅₀ values and the increase of resistance in the MTX-resistant variants were drawn from the MTX dose–response curve of each cell line (Table 1). The increase in MTX resistance compared with the parental cell line ranged from 3- to 135-fold for U-2OS variants, and from 15- to 281-fold for Saos-2 variants.

MTX-resistant cell lines did not show any cross-resistance with cisplatin and doxorubicin, the only exceptions were the Saos-2/MTX30 and Saos-2/MTX1 μg variants, which showed a low-level cross resistance to doxorubicin (Table 1). Cross-resistance to trimetrexate was present only in U-2OS MTX-resistant variants, with the only exception of U-2OS/MTX3. In general, trimetrexate was found to be more effective than MTX in all cell lines. In fact, the values of trimetrexate IC₅₀ were significantly lower than the corresponding MTX IC₅₀ values in all cell lines, with the only exceptions of U-2OS and U-2OS/MTX3, both of which were highly sensitive to both drugs.

**In vitro growth characteristics**

The analysis of in vitro growth characteristics of all cell lines showed that doubling time was significantly longer in all U-2OS MTX-resistant variants than in the parental cell line, with U-2OS/MTX3 the only exception, which showed a doubling time similar to that of U-2OS (data not shown). Saturation density was significantly decreased only in the Saos-2/MTX1 μg cell line, whereas no difference was found between MTX-resistant variants and their corresponding parental cell lines with respect to cloning efficiency (data not shown).

**Analysis of DHFR and RFC gene status in cell lines**

Analysis of DHFR mRNA levels showed a progressive increase in DHFR gene expression associated with MTX resistance in U-2OS but not in Saos-2 MTX-resistant variants (Figure 1). The increase of DHFR gene expression in U-2OS MTX-resistant variants ranged from 3.5- (U-2OS/MTX30) to 50.9-fold (U-2OS/MTX300). No evidence of DHFR gene amplification was found in Saos-2 cell lines (Figure 2). Similar results were obtained by Southern blot analysis (data not shown).

### Table 1. Sensitivity of the U-2OS, Saos-2 and MTX-resistant cell lines to anticancer drugs

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Methotrexate</th>
<th>Doxorubicin</th>
<th>Cisplatin</th>
<th>Trimetrexate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC₅₀, ng/ml (µM)</td>
<td>Fold increase</td>
<td>IC₅₀, ng/ml (µM)</td>
<td>Fold increase</td>
</tr>
<tr>
<td>U-2OS</td>
<td>3.5 ± 1.0 (0.01)</td>
<td>3.1 ± 1.1 (0.01)</td>
<td>110.3 ± 25.6 (0.37)</td>
<td>3.3 ± 2.1 (0.01)</td>
</tr>
<tr>
<td>U-2OS/MTX3</td>
<td>11.9 ± 3.2 (0.03)</td>
<td>3.3 ± 1.2 (0.01)</td>
<td>133.4 ± 32.4 (0.44)</td>
<td>7.3 ± 1.4 (0.01)</td>
</tr>
<tr>
<td>U-2OS/MTX30</td>
<td>72.3 ± 27.7 (0.16)</td>
<td>3.4 ± 1.3 (0.01)</td>
<td>152.0 ± 28.9 (0.51)</td>
<td>30.4 ± 6.2 (0.05)</td>
</tr>
<tr>
<td>U-2OS/MTX100</td>
<td>240.9 ± 33.4 (0.53)</td>
<td>3.8 ± 1.3 (0.01)</td>
<td>158.5 ± 30.1 (0.53)</td>
<td>79.5 ± 11.3 (0.14)</td>
</tr>
<tr>
<td>U-2OS/MTX300</td>
<td>471.0 ± 10.0 (1.04)</td>
<td>3.9 ± 1.5 (0.01)</td>
<td>160.2 ± 22.3 (0.53)</td>
<td>131.5 ± 15.3 (0.23)</td>
</tr>
<tr>
<td>Saos-2</td>
<td>13.3 ± 3.0 (0.03)</td>
<td>7.0 ± 0.9 (0.01)</td>
<td>109.9 ± 26.3 (0.37)</td>
<td>6.5 ± 2.1 (0.01)</td>
</tr>
<tr>
<td>Saos-2/MTX30</td>
<td>198.1 ± 28.1 (0.44)</td>
<td>7.2 ± 1.1 (0.01)</td>
<td>154.2 ± 76.8 (0.51)</td>
<td>7.4 ± 3.1 (0.01)</td>
</tr>
<tr>
<td>Saos-2/MTX100</td>
<td>316.1 ± 35.0 (0.70)</td>
<td>9.3 ± 1.3 (0.02)</td>
<td>165.5 ± 65.5 (0.55)</td>
<td>8.0 ± 3.7 (0.01)</td>
</tr>
<tr>
<td>Saos-2/MTX300</td>
<td>1454.2 ± 41.5 (3.20)</td>
<td>18.5 ± 0.9 (0.03)</td>
<td>177.8 ± 50.4 (0.59)</td>
<td>13.5 ± 1.0 (0.02)</td>
</tr>
<tr>
<td>Saos-2/MTX1µg</td>
<td>3740.0 ± 59.0 (8.23)</td>
<td>19.5 ± 1.5 (0.03)</td>
<td>190.8 ± 38.9 (0.64)</td>
<td>15.0 ± 2.7 (0.03)</td>
</tr>
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</table>

*All determinations were carried out in triplicate.

*IC₅₀ data refer to mean (ng/ml) ± standard error and µM refers to the IC₅₀ (ng/ml) mean values.

*Ratio between IC₅₀ (ng/ml) of MTX variants to IC₅₀ (ng/ml) of their corresponding parental cell line.
Quantitation of RFC mRNA revealed a remarkable decrease of RFC gene expression associated with the development of in vitro MTX resistance in Saos-2 MTX-resistant variants (Figure 3). The level of RFC gene expression started to decrease in Saos-2/MTX30 (0.34-fold compared with Saos-2) and progressively reached the lowest value in Saos-2/MTX1μg (0.04-fold compared with Saos-2). A less remarkable decrease of RFC expression was also present in U-2OS MTX-resistant variants (Figure 3).

Analysis of RFC gene copy number by dot blot and Southern blot did not show any difference between the parental cell lines and their MTX-resistant variants, indicating that the reduced RFC expression was not due to gene deletion (data not shown).

Figure 1. Expression levels of the DHFR gene in U-2OS and Saos-2 human osteosarcoma cell lines and their MTX-resistant variants, as evaluated by quantitative real-time RT-PCR. (A) Fluorescence versus cycle number plot of the DHFR transcript in U-2OS cell lines. Starting from the top, amplification profiles refer to the U-2OS/MTX300, U-2OS/MTX100, U-2OS/MTX30, U-2OS/MTX3 and U-2OS cell lines. (B) Fluorescence versus cycle number plot of DHFR transcript and amplification profiles in Saos-2 cell lines. (C) and (D) Fold-increase in DHFR expression of (C) U-2OS/MTX and (D) Saos-2/MTX resistant variants compared with the correspondent parental cell lines. Results represent the mean ± standard error of three independent experiments.

Figure 2. Assessment of DHFR gene amplification in the U-2OS and Saos-2 human osteosarcoma cell lines, and their MTX-resistant variants, by dot blot. The level of DHFR gene amplification of each MTX-resistant variant was determined in comparison with its corresponding parental cell line. Results shown are representative of three independent experiments.
decrease of RFC gene expression (Table 2). The presence of DHFR gene amplification (HOS and SARG cell lines), of reduced RFC gene expression (IOR/MOS) or of both mechanisms (IOR/OS18) paralleled the degree of MTX-unresponsiveness of these cell lines to MTX.

Assessment of DHFR, pRB and E2F-1 proteins

The assessment of DHFR cellular content by western blotting showed an evident increase in protein levels only in U2OS/MTX-resistant variants (Figure 4). In Saos-2 MTX-resistant variants, DHFR protein levels did not significantly increase along with the degree of MTX resistance.

Immunodetection of pRB by western blotting in MTX-resistant variants showed that only U2OS-derived cell lines expressed normal-sized pRB, which was absent in Saos-2 cells (data not shown). Indirect immunofluorescence further confirmed that all MTX-resistant variants retained the same characteristics as their corresponding parental cell line. A nuclear immunostaining for pRB was evident in all the U2OS cell lines, but in none of the Saos-2 cell lines (data not shown).

Assessment of pRB by western blotting in the four human osteosarcoma cell lines detected normal-size pRB in HOS, SARG and IOR/OS18 cell lines, but not in the IOR/MOS cell line (Table 2).

Table 2. DHFR and RFC gene status in relation to RB1 and resistance to MTX in four human osteosarcoma cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>pRB</th>
<th>DHFR copy number versus U2OS</th>
<th>RFC expression level versus U2OS</th>
<th>IC50 MTX, ng/ml (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOS</td>
<td>Positive</td>
<td>2.0</td>
<td>1.1</td>
<td>2.4 (0.01)</td>
</tr>
<tr>
<td>SARG</td>
<td>Positive</td>
<td>3.5</td>
<td>0.7</td>
<td>13.2 (0.03)</td>
</tr>
<tr>
<td>IOR/OS18</td>
<td>Positive</td>
<td>3.3</td>
<td>0.5</td>
<td>50.1 (0.11)</td>
</tr>
<tr>
<td>IOR/MOS</td>
<td>Negative</td>
<td>1.1</td>
<td>0.1</td>
<td>89.9 (0.20)</td>
</tr>
</tbody>
</table>

pRB, RB1 protein.

Analysis of DHFR and RB1 genes in osteosarcoma clinical samples

By using paraffin-embedded samples, only DNA and tumour tissue sections were available for the study of clinical samples, that included evaluation of DHFR gene copy number and assessment of pRB protein. Immunohistochemistry was chosen for the
A poor response to chemotherapy (percentage of tumour necrosis <90%) was found in 3/4 patients (75%) with RH1 status on the basis of findings which demonstrated that this is the most convenient and reliable method of detecting the presence of non-altered pRB in human osteosarcoma clinical specimens [24]. Due to the nature of the samples and the lack of specific antibodies for immunohistochemical detection of the proteins RFC and DHFR, no mRNA or protein expression studies for RFC and DHFR were performed.

For the assessment of DHFR gene copy number by dot blot, the results of densitometric analysis were referred to those obtained with the U-2OS cell line, which was used as an internal reference in each determination. An amplification of the DHFR gene >2-fold was found in 4/14 (29%) primary tumours (samples 6, 11, 13 and 14) and in 2/2 (100%) lung metastases (samples 15 and 16) (Figure 5). In the primary lesions showing increased DHFR gene copy number, the amplification of the DHFR gene was an inherent feature of the tumour because none of these patients had received chemotherapy before sampling. In one case, with available specimens of the primary tumour (sample 14) and the lung metastasis arisen after chemotherapy (sample 15), DHFR gene analysis showed a significantly higher level of DHFR amplification in the metastasis compared with the primary lesion (Figure 5).

The clinico-pathological characteristics of patients from whom the clinical samples were obtained and the immunohistochemistry results are summarised in Table 3. Using immunohistochemistry, 8/14 (57%) primary tumours and 2/2 lung metastases were found to be pRB positive. Among primary lesions, amplification of the DHFR gene was present in 3/8 pRB-positive cases (38%) and in 1/6 pRB-negative cases (17%), suggesting that an inherent DHFR amplification may occur more frequently in RH1-positive tumours (P = 0.58, two-tailed Fisher’s exact test).

A poor response to chemotherapy (percentage of tumour necrosis <90%) was found in 3/4 patients (75%) with DHFR amplification and in 3/10 patients (30%) without an increase of DHFR gene copy number (P = 0.24, two-tailed Fisher’s exact test).

**Discussion**

Development of drug resistance is a common clinical problem that significantly decreases the effectiveness of chemotherapy in human cancers. In particular, in high-grade osteosarcoma patients, responsiveness to chemotherapy is a critical factor that dramatically influences clinical outcome [7, 25–27]. Therefore, a better knowledge of the cellular mechanisms responsible for resistance to the drugs currently used in conventional chemotherapeutic regimens may be of great help in drawing alternative and more effective therapeutic approaches for unresponsive patients.

Although MTX is a leader drug, together with doxorubicin, in osteosarcoma chemotherapy [6, 28], the cellular basis of MTX resistance in osteosarcoma cells has not been fully characterised. On the basis of the only study reported so far [12], it appears that the decreased expression of the RFC gene and/or the increase of DHFR expression levels are the most important mechanisms responsible for the degree of clinical resistance to MTX in osteosarcoma patients. Therefore, the aim of this work was the evaluation of the specific role of RFC and DHFR genes in the development of MTX resistance in human osteosarcoma cells.

The RFC gene encodes the membrane carrier responsible for folate uptake, a carrier that is also used by antifolates, including MTX, to gain entry into cells [29]. Decreased RFC levels may therefore result in a less efficient intracellular transport of antifolates with a consequent reduction of their cytotoxicity.

DHFR is the major and most important target for MTX activity, and the concentration of MTX required to achieve inhibition of DHFR enzymatic activity is strictly dependent on the intracellular levels of this enzyme [9]. Like other cell cycle-related genes, expression of DHFR is negatively regulated by the RH1 gene, via E2F, and therefore RH1 can influence the cellular sensitivity to drugs targeting DHFR [11, 14–16, 30].

In our cell lines, resistance to MTX appeared to arise through partially, non-mutually exclusive, different pathways in relation to the RH1 gene status. In particular, the degree of MTX resistance in U-2OS (RH1-positive) cell lines was mainly associated with an increase in DHFR intracellular levels—as a result of amplification and consequent overexpression of the DHFR gene—together with a slight decrease in RFC gene transcriptional activity. On the other hand, in Saos-2 (RH1-negative) cell lines, development of MTX resistance was mostly associated with a decrease in expression of the RFC gene, without any significant involvement of DHFR.

In all these cell lines, the levels of E2F-1 were also determined, as it is one of the downstream targets of RH1 action that has been suggested to be responsible for increased DHFR expression and higher MTX resistance in several human sarcoma cell lines [16, 30, 31]. The assessment of E2F-1 protein by western blot did not reveal any difference between MTX-resistant variants and parental cell lines suggesting that, in agreement with reported data for other experimental models [23], E2F-1 is not involved in the development of MTX resistance in our cell lines.

Our findings indicate that MTX resistance may arise through partially different patterns in RH1-positive and RH1-negative
The analysis of $DHFR$ gene status and immunodetection of pRB in 16 osteosarcoma clinical samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Type</th>
<th>Hystotype</th>
<th>Histological grade</th>
<th>Site</th>
<th>Treatment before sampling</th>
<th>$DHFR$ gene*</th>
<th>pRB*</th>
<th>Response to chemotherapy*</th>
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<tr>
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<td>Tibia</td>
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<td>Positive</td>
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</table>

*Amplification of the $DHFR$ gene was defined in comparison with the U-2OS human osteosarcoma cell line.

†Immunohistochemistry for pRB.

‡Poor, tumor necrosis <90%; good, tumor necrosis >90%.

§Same patient.

Table 3. Analysis of $DHFR$ gene status and immunodetection of pRB in 16 osteosarcoma clinical samples

Cells. The U-2OS cells tend to amplify $DHFR$ in order to overcome the negative regulation of the $RB1$ gene on $DHFR$ gene expression and to achieve adequate levels of in vitro MTX resistance, which are partly also reached through the decreased expression of the $RFC$ gene. On the contrary, Saos-2 cells developed MTX resistance more quickly, as observed during the establishment of resistant variants, through a remarkable decrease in $RFC$ gene expression and without any significant involvement of $DHFR$.

Analysis of $DHFR$ and $RFC$ gene status in four additional human osteosarcoma cell lines revealed an inherent low-level amplification of $DHFR$ in the $RB1$-positive cell lines (HOS, SARG and IOR/OS18), and a remarkable inherent decrease of $RFC$ expression in the $RB1$-negative IOR/MOS cell line. In particular, the IOR/OS18 cell line showed the simultaneous presence of both mechanisms of MTX resistance, further suggesting that they are not completely mutually exclusive. In agreement with $DHFR$ or $RFC$ gene status, the three cell lines that showed either a clear $DHFR$ gene amplification (SARG), decreased $RFC$ expression (IOR/MOS) or both mechanisms (IOR/OS18) also presented a reduced sensitivity to MTX.

The analysis of a series of osteosarcoma clinical samples showed that, in agreement with the results obtained in our experimental models, the incidence of $DHFR$ amplification appears to be higher in pRB-positive cases. Moreover, our data indicate that amplification of the $DHFR$ gene can be present at clinical onset as an inherent feature of osteosarcoma cells and appears to be associated with a reduced response to chemotherapy. Although a confirmation of these data in large scale studies is warranted, our findings indicate that $DHFR$ gene amplification can be present at the time of diagnosis as an inherent feature of the tumour and may negatively influence the response to chemotherapy of osteosarcoma patients. In agreement with our findings, an inherent increase in $DHFR$ gene copy number was also previously found in samples derived from untreated soft-tissue sarcoma patients, in which low-level amplification of the $DHFR$ gene was suggested to be sufficient to determine the natural clinical resistance to MTX in these neoplasms [32].

Taken together, our data indicate that, in presence of the $RB1$-inhibitory effect on $DHFR$ gene expression, osteosarcoma cells could be more prompt to amplify the $DHFR$ gene than $RB1$-negative cells. The fact that an inherent low-level amplification of $DHFR$ was found only in the $RB1$-positive cell lines (HOS, SARG and IOR/OS18) and appeared to be more frequent in pRB-positive tumours supports this hypothesis. The reason for the inherent amplification of the $DHFR$ gene that we found in both osteosarcoma cell lines and in tumours of patients who were not previously treated with chemotherapy is still not clear but, most probably, it occurs since increased levels of DHFR are essential for tumour cell growth. However, this should be taken into account when planning alternative treatments with antifolate drugs targeting DHFR for MTX-unresponsive patients.

Among these antifolates, trimetrexate has recently been proposed in several tumours as a possible candidate for overcoming
the clinical resistance to MTX [18, 22]. Trimitrexate is a powerful inhibitor of DHFR that does not use RFC to enter the cell and does not require polyglutamation for intracellular retention. The effectiveness of trimitrexate has been evaluated in different tumours [11, 21, 22], but no data have been reported so far about its activity in osteosarcoma.

Data obtained in different experimental systems demonstrated that an increase in DHFR levels is the most important mechanism of trimetrexate resistance [19]. In agreement with these findings, analysis of trimetrexate efficacy in our experimental model revealed cross-resistance to this drug only in U-2OS MTX-resistant variants, which showed DHFR gene amplification and remarkably higher levels of DHFR protein. Moreover, trimitrexate was invariably more effective than MTX in almost all our MTX-resistant variants, indicating that it can be considered as a promising candidate for the treatment of MTX-unresponsive osteosarcoma patients. The efficacy against osteosarcoma cells of other antifolates, which target thymidylate synthase as opposed to DHFR—in addition to being active against cells with high DHFR contents (like raltitrexed or CB3771) [33]—will also be evaluated in order to find additional alternative therapeutic strategies for osteosarcoma patients showing increased DHFR levels.

In conclusion, our study indicates that the simultaneous evaluation at clinical onset of DHFR and RB1 gene status, as well as of RFC expression levels, may be useful in the identification of tumours resistant to MTX. This may be of help also for the identification of potentially MTX-unresponsive osteosarcoma patients who would benefit from alternative treatment protocols.

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References

27. Serra M, Scotlandi K, Reverter-Brenchat G et al. Value of P-glycoprotein and clinicopathologic factors as the basis for new treatment strategies in


