Clinical impact of the methotrexate resistance-associated genes C-MYC and dihydrofolate reductase (DHFR) in high-grade osteosarcoma

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Background: Aims of this study were the validation of C-MYC involvement in methotrexate (MTX) resistance and the assessment of clinical impact of C-MYC and dihydrofolate reductase (DHFR) in osteosarcoma (OS).

Materials and methods: The involvement of C-MYC in MTX resistance was validated with an antisense approach. C-MYC and DHFR protein levels at diagnosis were assessed by immunohistochemistry on series of patients treated with either a MTX-based protocol (IOR/OS-1; 72 patients) or with a standard four-drug regimen (ISG/SSG 1; 61 patients).

Results: Down-regulation of C-MYC significantly decreased the MTX resistance level of OS cells, demonstrating its causal involvement in this phenomenon. In clinical samples, a worse outcome was associated with increased levels of DHFR and C-MYC at diagnosis in the IOR/OS-1 patients and of C-MYC in the ISG/SSG 1 patients.

Conclusions: Meanwhile the adverse clinical impact of DHFR overexpression appeared to be closely related to the relevance of MTX in the chemotherapeutic protocol, that of C-MYC overexpression was more general and not strictly MTX related. The assessment of C-MYC and DHFR at diagnosis, together with that of other known prognostic markers, can be considered for an early identification of subgroups of OS patients with higher risk of adverse outcome.

Key words: C-MYC, DHFR, drug resistance, osteosarcoma, P-glycoprotein, prognostic markers

Introduction

Osteosarcoma (OS), the most common primary malignant tumour of bone, is characterised by an extremely high biologic aggressiveness with rapid development of metastases [1–3]. Despite the aggressive chemotherapeutic regimens that are currently used, treatment progress in OS seems to have reached an impasse and ~40% of patients acquire drug resistance and die of disease progression [3, 4].

Methotrexate (MTX) is widely used in multidrug chemotherapeutic regimens for high-grade OS, together with doxorubicin, cisplatin and ifosfamide [3–5]. However, although it is one of the most important drugs for OS treatment, the cellular mechanisms and genes associated with the development of MTX resistance in OS cells are still scarcely defined. Those that, in experimental models, have been demonstrated to be undoubtedly involved in MTX resistance are the increase of dihydrofolate reductase (DHFR) enzyme levels, due to DHFR gene amplification and/or overexpression [6, 7], and the impaired intracellular transport of MTX as consequence of decreased expression of the reduced folate carrier (RFC) gene [6–8]. However, in high-grade OS, only the clinical impact of RFC has been evaluated [8, 9], whereas data about the clinical relevance of DHFR are still very scarce and incomplete [7, 8].

In order to gather more detailed information on the genomic changes associated with MTX resistance in OS cells, we have carried out a genomic profiling of a series of human OS MTX-resistant cell lines in the past years [6]. By coupling data obtained from comparative genomic hybridisation on chromosomes and on microarrays, we have selected a small group of genes to be prioritised for further validations on our experimental models and, eventually, on clinical settings [6]. Apart from DHFR, the most relevant of these prioritised genes was C-MYC, of which copy number gain was found in several of our MTX-resistant cell lines and appeared to influence the inherent MTX sensitivity of human OS cell lines [6].

Since our previous findings did not clarify whether copy number gain of C-MYC was causally related to the development of MTX unresponsiveness, the first aim of this study was the validation of the involvement of C-MYC in the degree of resistance to MTX in our sets of MTX-resistant human OS cell lines. In the second phase of this study, the
clinical impact of C-MYC was assessed, together with that of DHFR, on adequate series of OS clinical samples, in order to establish their actual prognostic value in relation to the treatment protocol characteristics and clinical outcome. Finally, the prognostic value of both C-MYC and DHFR was analysed in comparison with that of P-glycoprotein, a well-known adverse prognostic marker related to drug resistance in high-grade OS [10].

materials and methods

drugs and cell lines

MTX-resistant variants of the U-2OS and Saos-2 human OS cell lines were obtained and maintained in culture as previously described [7].

RNA isolation and quantitative real-time polymerase chain reaction

Total cellular RNAs were extracted from cell pellets obtained by using the TRizol reagent (Invitrogen, Milan, Italy), according to standard procedures. For real-time polymerase chain reaction (PCR), 500 ng of total RNA was reverse transcribed into complementary DNA (cDNA) at 37°C for 2 h and gene expression analyses were carried out by using SYBR® Premix Ex Taq™ (Takara, Shiga, Japan). The gene targets were amplified with the following primers: C-MYC-forward CGTCCCTGGATCTCTGCTCTC; C-MYC-reverse GCTGCGTAGTTGTGCTGATGTG; DHFR-forward ATTTCCAGAGAATGACCACAACC; DHFR-reverse ACAGAAGCTGCAACCATATCC. PCR amplifications were carried out with the ABI PRISM 7900 SDS (Applied Biosystems, Foster City, CA for 40 cycles (95°C for 10 s, 60°C for 20 s, 72°C for 30 s) after an initial incubation step at 95°C for 10 s. For each sample, the PCR reaction was carried out in triplicate. Reactions omitting enzyme or RNA were used as negative controls. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (amplified with the TTGAAGTGACAGGACAGA CCGTGATG; forward and CACACCATGGAAGGCTGGGCT-reverse primers) was used as housekeeping gene. GAPDH delta Ct values were used to normalise all other genes tested from the same cDNA sample. The fold differences of target gene expression in MTX-resistant variants compared with their parental cell lines were calculated with the comparative Ct method (∆∆Ct method).

western blot

Cultured cells were briefly washed and collected by scraping in cold phosphate-buffered saline solution. Nuclear proteins were extracted with the Nuclear Extract Kit (Active Motif, Carlsbad, CA) according to the manufacturer’s instructions. Western blot analyses were carried out by using standard methods with the anti-C-MYC 9E10 mouse mAb (Oncogene Research Products, Manhasset, NY) at a 0.4 µg/ml concentration. After hybridisation with the anti-C-MYC mAb, membranes were stripped and then rehybridised with the anti-histone H1 mouse mAb (Abcam, Cambridge, UK) at 0.5 µg/ml concentration, in order to quantify the protein loading of each sample. Protein bands were visualised using an enhanced chemiluminescence detection system (Immobilon Western, Millipore, Billerica, MA according to manufacturer’s protocols. For each sample, the amount of C-MYC protein relative to that of histone H1 was determined by densitometric analysis.

C-MYC antisense treatment

The 15-mer antisense phosphorothioate oligodeoxynucleotide (ODN), which is complementary to the translation initiation region of C-MYC mRNA (59-AAAGTTGAGGGGCAT-39), and the control scrambled sequence ODN containing the ‘G-quartet’ motif (59-AAAGCATAGGGGGTGT-39) were produced by INEX Pharmaceuticals (Vancouver, British Columbia, Canada) and kindly provided to us by Dr Carlo Leonetti (Regina Elena Cancer Institute, Rome, Italy).

Antisense experiments were carried out on the two variants with the highest amount of C-MYC protein inside each series, namely U-2OS/MTX300 and Saos-2/MTX300. For each cell line, 2·10⁵ cells were seeded in 60 mm Petri dishes in drug-free Iscove’s Modified Dulbecco’s medium (IMDM; Invitrogen Ltd, Paisley, UK) plus 10% foetal bovine serum (FBS; Biowhittaker Europe, Cambrex-Verviers, Belgium) without antibiotics. After 24 h, medium was changed and cell cultures were incubated with Lipofectamine Plus reagent (Invitrogen Italia) dissolved in serum-free, drug-free OptiMem medium, without antibiotics but with C-MYC antisense or scrambled ODNs. Controls were cultured in the same media without ODNs. Incubation with C-MYC antisense or scrambled ODNs was carried out for 2 h in U-2OS/MTX300 and for 12 h in Saos-2/MTX300 cells, respectively. These incubation times were defined on the basis of the evidence obtained from liposomes incorporation experiments with fluorescein-conjugated ODNs. After transfection, culture medium was replaced with IMDM 10% FBS plus 300 ng/ml MTX and cells were harvested at different time points to assess gene and protein expression levels, as well as growth inhibition and apoptosis induction. C-MYC gene expression was analysed by quantitative real-time PCR as described above. C-MYC protein levels were assessed by using the QuantiSirt specific gene knock-down quantification kit (Epigentek, Brooklyn, NY), an ELISA-based assay which is more sensitive than western blot, by following manufacturer’s procedures. The extent of growth inhibition in comparison with controls was estimated by using the Trypan dye exclusion method. Detection and quantification of apoptotic cells was carried out by the annexin-V assay by using the MBL MEBCYTO Apoptosis kit (Medical and Biological Laboratories, Naka-ku Nagoya, Japan) according to the manufacturer’s instructions.

immunohistochemistry

Immunohistochemistry was carried out on series of paraffin-embedded tissue samples obtained at diagnosis from high-grade OS patients, which were homogeneously treated with either the IOR/OS1 protocol, a MTX-based chemotherapy regimen [11], or with the ISG/SSG 1 protocol, an Italian/Scandinavian treatment protocol based on the administration of doxorubicin, MTX, cisplatin and ifosfamide [12]. Only Italian patients were included in the ISG/SSG 1 protocol. The eligibility criteria for both protocols were as follows: diagnosis of central high-grade OS of the extremity, age younger than 40 years, absence of metastases at the time of diagnosis and no prior chemotherapy or surgical treatment for bone lesions. In the IOR/OS-1 treatment protocol, all patients were preoperatively treated with two cycles of MTX, administered i.v. either at high (7500 mg/m²) or moderate doses (750 mg/m²), and two cycles of cisplatin, administered...
In the postoperative phase, patients with a good histological response (extent of tumour necrosis > 90%) received the same drugs and dosage used preoperatively. Patients with a fair histological response (extent of tumour necrosis between 60% and 89%) received the same drugs used preoperatively with the addition of doxorubicin. Patients with a poor histological response (tumour necrosis < 60%) received the same drugs used preoperatively with the addition of a combination of doxorubicin and bleomycin.

In the ISG/SSG 1 treatment protocol, preoperative chemotherapy consisted of two blocks of high-doses MTX, high-doses ifosfamide, cisplatin and doxorubicin. Postoperatively, MTX and ifosfamide were administered as in the preoperative treatment, doxorubicin dose was increased in all patients, whereas cisplatin dose was increased only in patients who did not show a total or almost total tumour necrosis. All drugs were administered i.v. After the end of chemotherapy, all patients were continuously followed and clinical data updated. Adverse events were defined as recurrence of the tumour at any site or death during remission. Event-free survival was calculated from the first day of chemotherapy until the date of first relapse, tumour-related death or last follow-up examination. Informed consents were collected from patients in accordance with the standard procedure of our Institute.

Tumour samples for immunohistochemical analyses were available from biopsy specimens of 72 IOR/OS-1 and 61 ISG/SSG 1 patients. The IOR/OS-1 series included 37 patients who were treated with high doses of MTX (7500 mg/m²) and 35 patients who received moderate doses of MTX (750 mg/m²), according to this treatment protocol. In addition to these two series, 29 primary tumour samples from OS patients with metastasis at diagnosis were also analysed, in order to verify whether any marker expression was associated with a different inherent biologic tumour aggressiveness.

All immunohistochemical assessments were carried out by using an avidin–biotin peroxidase complex method (Vectastain ABC kit, Vector Laboratories, Burlingame, CA). Expression of DHFR was evaluated with the anti-DHFR, clone 49 mouse mAb (BD PharMingen, San Diego, CA). To define the optimal dilution rate to be used on clinical samples, we assessed the antibody on formalin-fixed, paraffin-embedded sections of cell pellets obtained from U-2OS and U-2OS/MTX30 cell lines. The 10 μg/ml antibody concentration, which was used for immunohistochemical analysis on clinical samples, was chosen in order to reveal only DHFR levels similar or higher to those of the U-2OS/MTX30 paraffin-embedded cells.

Expression of C-MYC was evaluated with the anti-MYC 9E10 mouse mAb (Oncogene Research Products) at a 4 μg/ml concentration, after antigen retrieval with microwave at 750 W (three cycles of 5 min each) in a citrate buffer solution (containing 0.01 mol/l citric acid and 0.01 mol/l sodium citrate, pH 6.0).

Tissue sections were incubated with primary antibodies at 4°C overnight for C-MYC and for 48 h for DHFR. The final reaction product was revealed by incubation with diaminobenzidine (Sigma, St Louis, MO) and nuclei were counterstained with Gill’s haematoxylin (Sigma). Negative controls were carried out by replacing the primary antibody with normal horse serum. Moreover, for each case, one additional section was immunostained with the V9 anti-vimentin mAb (Roche Molecular Biochemicals, Mannheim, Germany), in order to control the antigenicity preservation of tumour specimen. Samples negative for vimentin were not considered as eligible for the immunohistochemical study.

Evaluation of samples was carried out in a blinded fashion, without knowing the clinical outcome of patients. For DHFR, only specimens with a positive immunostaining in >10% of tumour cells, regardless of the staining intensity, were considered as positive. For C-MYC, only tumour samples showing nuclear immunostaining were considered as positive.

In the series of IOR/OS-1 cases, in addition to DHFR and C-MYC, we also assessed the expression of P-glycoprotein as previously described [13, 14].

In the ISG/SSG 1 treatment protocol, preoperative chemotherapy consisted of two blocks of high-doses MTX, high-doses ifosfamide, cisplatin and doxorubicin.

In the ISG/SSG 1 treatment protocol, preoperative chemotherapy consisted of two blocks of high-doses MTX, high-doses ifosfamide, cisplatin and doxorubicin.
controls (Figure 2).

in cells treated with scrambled ODNs compared with the growth inhibition and apoptosis induction was ever observed significant C-MYC gene expression or protein level changes, antisense-treated cells (data not shown). No evidence of U-2OS/MTX300 and at 48 h in Saos-2/MTX300 ODN were also observed, although at a less extent, at 6 h in (Figure 2C and D). Growth inhibition and apoptosis induction in ODN antisense-treated cells compared with controls were also present induced growth inhibition and apoptosis in ODN antisense-treated cells. At these time points, the maximum effects on MTX-induced growth inhibition and apoptosis in ODN antisense-treated cells with controls were also present (Figure 2C and D). Growth inhibition and apoptosis induction were also observed, although at a less extent, at 6 h in U-2OS/MTX300 and at 48 h in Saos-2/MTX300 ODN antisense-treated cells (data not shown). No evidence of significant C-MYC gene expression or protein level changes, growth inhibition and apoptosis induction was ever observed in cells treated with scrambled ODNs compared with the controls (Figure 2).

immunohistochemistry

The clinical series considered for the immunohistochemical analyses included 72 patients treated with the IOR/OS-1 protocol, 61 patients treated with the ISG/SSG 1 protocol and 29 patients with metastatic OS at diagnosis. Data concerning P-glycoprotein expression in the ISG/SSG 1 patients included in this study were derived from those which were recently published [14]. Immunohistochemical positivity appeared to be clearly localised in the cytoplasm for DHFR and in the nucleus of tumour cells for C-MYC (Figure 3).

As shown in Table 1, no statistically significant difference was found in the incidence of positivity to DHFR, C-MYC or P-glycoprotein between nonmetastatic and metastatic patients. Inside each group, the presence of positivity to one of these three proteins resulted to be independent from the other two, indicating that they were not correlated (data not shown). The lack of correlation between the presence of immunohistochemical positivity to these three proteins was present also when IOR/OS-1 and ISG/SSG 1 patients were considered all together (data not shown).

In the groups of IOR/OS-1 or ISG/SSG 1 patients, we also analysed the relationship between the immunohistochemical positivity to each marker and the most relevant clinicopathologic features for high-grade OS (gender, age, tumour location, histologic subtype, tumour volume at diagnosis, type of surgery and surgical margins). No evidence of significant correlations between any immunohistochemical marker and these clinicopathologic features was found (data not shown). Moreover, concerning parameters related to chemotherapy response, no significant correlation was found between any marker expression and tumour necrosis in the ISG/SSG 1 protocol. Relationship with tumour necrosis was not analysed in the IOR/OS-1 protocol since the good response to preoperative treatment was probably overestimated because of the intraarterial infusion of cisplatin, which increases the effects on tumour necrosis due to the local higher drug concentrations independently from the actual tumour responsivity to chemotherapy [15, 16].

relationship with clinical outcome

The prognostic value of each marker and clinicopathologic variable was analysed in relation to relapse rate and clinical outcome inside each protocol. Relapse occurred in 38 of 72 IOR/OS-1 patients (53%) and in 23 of 61 ISG/SSG 1 patients (38%). All adverse events occurred within the first 5 years of follow-up, with the only exception of one IOR/OS-1 patient who relapsed at the 10th year. As shown in Table 2, a higher incidence of relapse was significantly associated with DHFR positivity in the IOR/OS-1 protocol and with C-MYC- or P-glycoprotein positivity in the ISG/SSG 1 protocol.

No significant associations were found between any clinicopathologic parameter and incidence of relapse in both protocols (data not shown).

According to the findings concerning relapse rate, event-free survival analyses of IOR/OS-1 patients further confirmed the association of DHFR positivity with a worse clinical outcome (Figure 4A). A worse event-free survival probability was also found to be significantly associated with C-MYC positivity (Figure 4B), in agreement with the trend found for relapse rate, but not with P-glycoprotein positivity (Figure 4C).

Event-free survival analyses of ISG/SSG 1 patients showed that meanwhile DHFR positivity lost the adverse prognostic value exhibited in the IOR/OS-1 protocol (Figure 4D), positivity to C-MYC (Figure 4E) or P-glycoprotein (Figure 4F) was significantly associated with a worse event-free survival.

None of the other markers or clinicopathologic parameters considered (gender, age, tumour location, histologic subtype, tumour volume at diagnosis, type of surgery and surgical
margins) resulted to be significantly associated with event-free survival, either in the IOR/OS-1 or in the ISG/SSG 1 protocol (data not shown).

Event-free survival analysis carried out in the two subgroups of IOR/OS-1 patients who received either moderate or high doses MTX showed that the increase of MTX dosage was able to improve the outcome of DHFR-negative patients only (supplementary figure 1, available online).

Multivariate analyses were carried out by considering the parameters that resulted to be significantly associated with clinical outcome by the univariate approach (Table 3). In the IOR/OS-1 treatment protocol, Cox’s proportional hazards regression analysis applied to event-free survival indicated a significant association with a higher risk ratio for adverse events for DHFR-positive patients. C-MYC-positive patients showed a trend towards an increased risk for adverse events,

Figure 2. Efficacy of C-MYC antisense oligonucleotides (ODNs) treatment in U-2OS/MTX300 and Saos-2/MTX300 variants. Determinations were repeated three times and data of one representative experiment are shown. Panels A and B show the C-MYC gene expression level assessed by quantitative real-time polymerase chain reaction in control (CTRL), antisense ODN-treated (AS) and scrambled ODN-treated (SCR) cultures. Data of AS or SCR cultures were referred to those of the controls. Panels C and D show the extent of MTX-induced growth inhibition and apoptosis (on the left side) and of the decrease of C-MYC protein levels (on the right side) compared with controls at the time points in which the maximum C-MYC protein down-regulation was reached (12 h for U-2OS/MTX300 and 72 h for Saos-2/MTX300).

Figure 3. Immunohistochemical detection of DHFR (A) and C-MYC (B) in a tumour tissue sample of high-grade osteosarcoma. Localisation of immunoreaction was cytoplasmic for DHFR and nuclear for C-MYC. Original magnification ×200.
Resistance to chemotherapeutic agents is a major obstacle for successful treatment of many tumours, including high-grade OS [4, 17]. One of the most important and widely used drugs for the chemotherapy of high-grade OS is MTX [2–4] and we found that, in addition to RFC and DHFR, also the C-MYC oncogene appeared to play an important role in MTX resistance development of human OS cells [6, 7]. However, meanwhile the causal association of RFC or DHFR with resistance to MTX was already confirmed, that of C-MYC was a new finding, which needed to be further analysed and validated. Therefore, the first aim of this study was the validation of C-MYC involvement in the development of MTX resistance in our series of MTX-resistant human OS cell lines.

In agreement with our previous indications [6], in this study we confirmed the presence of increased C-MYC expression and protein level in the majority of our MTX-resistant variants. The fold increase in C-MYC protein level resulted to be more evident in U-2OS/MTX-resistant variants, most probably because of the lower inherent C-MYC levels of U-2OS compared with Saos-2 parental cell line.

The validation of the causal C-MYC involvement in MTX resistance was carried out by treating the two variants with the highest fold change of C-MYC protein levels in comparison with their parental cell line (U-2OS/MTX300 and Saos-2/MTX300) with antisense ODNs. In both variants, antisense treatment was able to down-regulate C-MYC at both gene expression and protein level and, consequently, to remarkably decrease MTX resistance, as demonstrated by the appearance of remarkable collateral MTX-induced growth inhibition and apoptosis. Taken together, these data clearly demonstrated the causal involvement of C-MYC up-regulation in the acquisition of MTX resistance in our experimental models.

On the basis of these findings, the second phase of this study focused on the assessment of the clinical impact of C-MYC in adequate series of OS clinical samples, obtained from patients treated with chemotherapeutic protocols in which MTX played a relevant role. In addition to C-MYC, we also evaluated the clinical relevance of DHFR protein overexpression since data about its actual impact on clinical outcome of high-grade OS patients are still very scarce and incomplete [7, 8]. Finally, the prognostic value of C-MYC and DHFR was compared with that of P-glycoprotein, a well-known adverse prognostic marker related to drug resistance and clinical outcome in high-grade OS [10]. RFC protein, whose clinical relevance in high-grade OS was already determined [8], was not included in these analyses because it is almost impossible to reliably assess its decrease by immunohistochemistry due to the low basal RFC protein expression level which is at the limit of immunohistochemical detection [9].

Immunohistochemical analyses were carried out on different series of homogeneously treated high-grade OS patients, which underwent either the IOR/OS-1 (72 patients) or the ISG/SSG 1 (61 patients) chemotherapy protocol. We decided to consider these two different protocols in order to evaluate the clinical relevance of each marker in relation to the impact of MTX in the chemotherapeutic regimen. In fact, meanwhile the IOR/OS-1 was an MTX-based chemotherapy protocol, the ISG/SSG 1 was one of the currently used four-drug regimens (including doxorubicin, MTX, cisplatin and ifosfamide), in which the impact of MTX was still very important but less relevant than in the IOR/OS-1. Moreover, in order to evaluate whether any of the markers considered here was associated with a different inherent biologic aggressiveness of OS tumour cells, a group of 29 biopsies obtained from patients with detectable lung metastasis at diagnosis were also included in this phase of the study.

The absence of differences concerning the incidence of C-MYC- or DHFR-positivity in the IOR/OS-1 or ISG/SSG 1 patients compared with patients with metastasis at diagnosis, indicated that the inherent increased levels of these proteins were not related to a higher inherent biologic aggressiveness of human OS cells. The same evidence was found for P-glycoprotein-positivity, which was even less frequent in

### Table 1. Positivity to DHFR, C-MYC and P-glycoprotein in the clinical samples obtained from high-grade osteosarcoma patients treated with the IOR/OS-1 or ISG/SSG 1 protocols and from patients with evidence of metastasis at diagnosis (OS-met)

<table>
<thead>
<tr>
<th>Marker</th>
<th>Positive cases/total (%)</th>
<th>IOR/OS-1</th>
<th>ISG/SSG 1</th>
<th>OS-met</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHFR</td>
<td>22/72 (31%)</td>
<td>16/61 (26%)</td>
<td>9/29 (31%)</td>
<td></td>
</tr>
<tr>
<td>C-MYC</td>
<td>22/72 (31%)</td>
<td>19/61 (31%)</td>
<td>7/29 (24%)</td>
<td></td>
</tr>
<tr>
<td>P-glycoprotein</td>
<td>29/72 (40%)</td>
<td>32/61 (52%)</td>
<td>10/29 (34%)</td>
<td></td>
</tr>
</tbody>
</table>

DHFR, dihydrofolate reductase.

### Table 2. Relapse rates in high-grade osteosarcoma patients treated with the IOR/OS-1 or the ISG/SSG 1 chemotherapy protocols in relation to the immunohistochemical positivity to DHFR, C-MYC or P-glycoprotein

<table>
<thead>
<tr>
<th>Marker</th>
<th>Relapse rate (number of relapsed cases/total)</th>
<th>IOR/OS-1</th>
<th>ISG/SSG 1</th>
<th>P value&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHFR</td>
<td>Positive 73% (16/22)</td>
<td>0.04</td>
<td>44% (7/16)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Negative 44% (22/50)</td>
<td>36% (16/45)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-MYC</td>
<td>Positive 68% (15/22)</td>
<td>63% (12/19)</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Negative 46% (23/50)</td>
<td>26% (11/42)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-glycoprotein</td>
<td>Positive 62% (18/29)</td>
<td>53% (17/32)</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Negative 47% (20/43)</td>
<td>21% (6/29)</td>
<td></td>
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</tr>
</tbody>
</table>

<sup>a</sup>Only significant <i>P</i> values by two-tailed Fisher’s exact test have been reported.

DHFR, dihydrofolate reductase.
metastatic patients, in agreement with our previously reported observations [18].

The analysis of relationships with clinical outcome revealed that a higher incidence of relapse was significantly associated with DHFR positivity in the IOR/OS-1 protocol and with positivity to C-MYC or P-glycoprotein in the ISG/SSG 1 protocol. C-MYC positivity appeared to be also associated with a higher relapse rate in IOR/OS-1 patients, although this correlation did not reach statistical significance. In partial agreement with the impact on relapse, event-free survival analyses indicated that a worse outcome was significantly associated with positivity to C-MYC or DHFR in the IOR/OS-1 protocol and to C-MYC or P-glycoprotein in the ISG/SSG 1 protocol. Multivariate analyses further supported this evidence, although the higher risk ratio associated with C-MYC positivity did not reach statistical significance.

Taken together, our results indicated that, although all these three markers had prognostic value in high-grade OS, their clinical impact varied according to the relative relevance of the different drugs which were used in the chemotherapeutic protocol. In fact, meanwhile the impact of DHFR on clinical outcome resulted to be strictly related to the relevance of MTX in the chemoterapeutic protocol, that of C-MYC appeared to be more general. In other words, despite we demonstrated the causal association of C-MYC with MTX resistance development in human OS cell lines, the clinical impact of this oncogene appeared to be present also in standard OS chemotherapeutic protocols, which are not specifically based on MTX. The fact that P-glycoprotein resulted to have an adverse prognostic value only for ISG/SSG 1 patients confirmed the previously reported evidence for its relationship with the impact of doxorubicin in the chemotherapeutic treatment [14, 19].

Table 3. Cox’s proportional hazards regression analysis in 72 IOR-OS1 and 61 ISG/SSG 1 osteosarcoma patients adjusted for the markers and clinicopathologic parameters that resulted to be significantly related to clinical outcome by univariate analyses

<table>
<thead>
<tr>
<th>Treatment protocol and variables</th>
<th>Event-free survival</th>
<th>Adjusted risk-rate ratio (95% confidence interval)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IOR-OS1 DHFR</td>
<td></td>
<td></td>
<td>0.047</td>
</tr>
<tr>
<td>Positive</td>
<td></td>
<td>2.0 (1.0–3.8)</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td></td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>C-MYC</td>
<td></td>
<td></td>
<td>0.153</td>
</tr>
<tr>
<td>Positive</td>
<td></td>
<td>1.6 (0.8–3.2)</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td></td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>ISG/SSG 1 P-glycoprotein</td>
<td></td>
<td></td>
<td>0.045</td>
</tr>
<tr>
<td>Positive</td>
<td></td>
<td>2.6 (1.0–6.8)</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td></td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>C-MYC</td>
<td></td>
<td></td>
<td>0.021</td>
</tr>
<tr>
<td>Positive</td>
<td></td>
<td>2.7 (1.2–6.2)</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td></td>
<td>1.0</td>
<td></td>
</tr>
</tbody>
</table>

Significant P values are in bold.

DHFR, dihydrofolate reductase.
The strict association between the clinical relevance of DHFR and the impact of MTX in the chemotherapeutic treatment is also demonstrated by the observation that in the IOR/OS-1 protocol, the increase of MTX dosage was able to positively influence the outcome of DHFR-negative patients only. This finding indicated that, in a MTX-based chemotherapy protocol, the presence of DHFR positivity at diagnosis is invariably associated with an adverse prognosis, also when high doses MTX were used. This is an important information that should be taken into account, in particular, when patients are treated with MTX-based chemotherapy protocols, which are still used in several centres [20, 21] or when regimens with increased MTX dosage are recommended [22, 23].

In conclusion, the demonstration of the involvement of C-MYC in experimental and clinical drug resistance of OS is a new important evidence provided by our study, which, however, needs further investigations. The role of C-MYC in the response to antineoplastic drugs of human tumours is controversial. In some neoplasms, overexpression of C-MYC increases sensitivity to antineoplastic drugs by activating the apoptotic programme, whereas in other tumours the activation of C-MYC may play an important role in tumour progression of high-grade OS [25], but very few data have been reported so far concerning its possible involvement in drug resistance of this tumour. Xie et al. [26] have recently reported the evidence of a possible role of C-MYC in overexpression in reducing drug sensitivity of a human OS cell line. In particular, these authors demonstrated that by treating C-MYC-transfected MG-63 cells with C-MYC antisense oligonucleotides, it was possible to increase their in vitro sensitivity to cisplatin, indicating a possible association between C-MYC down-regulation and enhancement of drug sensitivity.

At present, we do not have additional information about the mechanisms through which C-MYC may influence the drug responsiveness of human OS cells and additional studies are presently ongoing to clarify in more detail the molecular mechanisms that are responsible for the C-MYC involvement in drug resistance of OS cells. Preliminary data obtained on a series of doxorubicin- and cisplatin-resistant human OS cell lines established in our laboratory have indicated that also these drug-resistant variants exhibit increased levels of C-MYC protein compared with their parental cell lines. Although these preliminary findings need further confirmation, they suggest that the apparent lack of correlation between the in vitro and the clinical results found in the present study may be due to the fact that the validation of C-MYC was completed only in MTX-resistant cell lines, the variants in which we originally reported the alteration of this oncogene [6]. These ongoing studies may also lead to the identification of new candidate prognostic or therapeutic markers since C-MYC or its downstream regulated genes represent an attractive target for cancer therapeutic purposes [27].

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references


