Sequence Alterations in the Reduced Folate Carrier Are Observed in Osteosarcoma Tumor Samples

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ABSTRACT

High-dose methotrexate is a standard component of therapy for high-grade osteosarcoma. Its effectiveness may be limited by intrinsic and acquired resistance. Decreased reduced folate carrier (RFC) expression has been shown in approximately half of osteosarcomas at diagnosis. Mutations and polymorphisms in the RFC gene have been reported in various cell lines. The purpose of this study was to investigate sequence alterations in the RFC gene in osteosarcoma tumor samples. The entire coding region of the RFC gene in samples from 162 osteosarcoma patients was screened by DNA single-stranded conformational polymorphism, followed by direct sequencing of single-stranded conformational polymorphism patterns in multiple samples. Eight samples had altered single-stranded conformational polymorphism patterns in exon 3 that were associated with nucleotide changes that altered the amino acid sequence. All of these RFC sequence variants appeared to be heterozygous. Heterozygous C/T and homozygous C also were observed at RFC cDNA position 790 in exon 3, which does not alter the amino acid coding sequence. This study shows that RFC sequence alterations are frequent in samples from osteosarcoma patients. Additional studies are under way to determine the clinical significance of these sequence alterations and their effect on methotrexate transport and resistance.

INTRODUCTION

Osteosarcoma is the most common primary malignant bone tumor, seen mostly in children and young adults. The identification of effective chemotherapy for patients with it has led to significant improvements in patient outcomes over the last several decades (1, 2). Current osteosarcoma treatment requires systemic chemotherapy and definitive resection of the primary tumor and all clinically detectable metastases. High-dose methotrexate with leucovorin rescue is a major component of therapy in most current protocols for osteosarcoma (1–5). Responses to conventional-dose methotrexate in osteosarcoma are less frequent and less complete than those seen in other malignancies, such as acute lymphocytic leukemia, in which methotrexate is used routinely in conventional doses (6, 7). The improved efficacy of high-dose compared with conventional-dose methotrexate suggests that osteosarcoma may have intrinsic methotrexate resistance (8, 9). Methotrexate is mainly transported into cells through the RFC, which functions as a bidirectional anion exchanger with a high affinity for reduced folates and methotrexate but a low affinity for folic acid (10). In osteosarcoma, preliminary data suggest the basis of intrinsic methotrexate resistance might be impaired transport. Approximately half of...

(9.2%) samples were identified with other RFC sequence variants in exon 2, none of which have been reported. The sequence variants in exon 2 included a G to A substitution at cDNA position 231, a G to A substitution at cDNA position 155, a C to T substitution at cDNA position 114, and a T to C substitution at cDNA position 104, resulting in a serine to asparagine substitution at amino acid 46, a glutamate to lysine substitution at amino acid 21, an alanine to valine substitution at amino acid 7, and a serine to proline substitution at amino acid 4, respectively. A deletion of A at cDNA position 126 resulting in a frameshift was also observed. Some of these variants were observed in multiple samples. Eight samples had altered single-stranded conformational polymorphism patterns in exon 3 that were associated with nucleotide changes that altered the amino acid sequence. All of these RFC sequence variants appeared to be heterozygous. Heterozygous C/T and homozygous C also were observed at RFC cDNA position 790 in exon 3, which does not alter the amino acid coding sequence. This study shows that RFC sequence alterations are frequent in samples from osteosarcoma patients. Additional studies are under way to determine the clinical significance of these sequence alterations and their effect on methotrexate transport and resistance.

Received 5/17/02; revised 9/6/02; accepted 9/10/02.

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1 Supported in part by Grant R01 CA-83132 from the National Cancer Institute and the National Children’s Cancer Foundation. Some patient samples were procured and distributed by the Cooperative Human Tissue Network.

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3 The abbreviations used are: RFC, reduced folate carrier; SSCP, single-stranded conformational polymorphism.
Reduced Folate Carrier Alterations in Osteosarcoma

Several mutations and polymorphisms of the RFC gene have been identified in cell lines that result in antifolate resistance. A polymorphism has been reported at amino acid 27 of the human RFC (12, 13), which will be discussed further in the “Discussion.” Several mutations have been reported in the RFC gene in antifolate-selected cell lines (Table 1; Refs. 14–25). Clusters of point mutations were observed in human and murine cell lines (21, 26), which might imply the presence of a selection advantage for these mutations.

Table 1 Mutations in RFC reported in prior studies

<table>
<thead>
<tr>
<th>Species</th>
<th>Cell lines</th>
<th>Nucleotide change(s)</th>
<th>Amino acid substitution</th>
<th>Antifolate resistance</th>
<th>Selection method</th>
<th>Ref. no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>MOLT-3/MTX 10,000</td>
<td>G168A, C212T</td>
<td>Codon 29, premature stop</td>
<td>10,000-fold to MTX</td>
<td>MTX selection</td>
<td>14</td>
</tr>
<tr>
<td>Murine</td>
<td>L1210-G5</td>
<td>C926T</td>
<td>Codon 122, premature stop</td>
<td>44-fold to MTX</td>
<td>MTX selection</td>
<td>15</td>
</tr>
<tr>
<td>Murine</td>
<td>L1210-MTXrA</td>
<td>G429C</td>
<td>Codon 171, premature stop</td>
<td>100-fold to MTX</td>
<td>MTX selection</td>
<td>16</td>
</tr>
<tr>
<td>Murine</td>
<td>L1210-Gla</td>
<td>G260A</td>
<td>Codon 109, premature stop</td>
<td>10-fold to MTX</td>
<td>MTX selection</td>
<td>17</td>
</tr>
<tr>
<td>Human</td>
<td>CEM/MTX</td>
<td>G227A</td>
<td>Codon 99, premature stop</td>
<td>200-fold to MTX</td>
<td>MTX selection</td>
<td>18</td>
</tr>
<tr>
<td>Human</td>
<td>CEM/MTX R1-3</td>
<td>C389T</td>
<td>Codon 100, premature stop</td>
<td>14,000–300,000-fold to MTX</td>
<td>Continuous exposure to MTX</td>
<td>19</td>
</tr>
<tr>
<td>Human</td>
<td>CEM/MTX1</td>
<td>G224A, G474A, CATG insertion at cDNA position 285</td>
<td>644R, S127N frameshift</td>
<td>243-fold to MTX</td>
<td>Continuous exposure to MTX</td>
<td>20</td>
</tr>
<tr>
<td>Human</td>
<td>GW70</td>
<td>G179C, G227A, G231T</td>
<td>V29L, E45K, S46I</td>
<td>100-fold to GW1843 and MTX</td>
<td>Continuous exposure to GW1843U89</td>
<td>21</td>
</tr>
<tr>
<td>Human</td>
<td>GW70/LF</td>
<td>G179C, G227A, G231T, G356C</td>
<td>V29L, E45K, S46I, D88H</td>
<td>100-fold to GW1843 and MTX</td>
<td>GW1843U89 selection, folic acid depletion</td>
<td>21</td>
</tr>
<tr>
<td>Hamster</td>
<td>MTXRII OuaR2-4</td>
<td>G1033A and an intron splicing mutation</td>
<td>G345R, premature stop</td>
<td>300-fold to MTX</td>
<td>Continuous exposure to MTX</td>
<td>22</td>
</tr>
<tr>
<td>Murine</td>
<td>L1210-D3</td>
<td>A142T, T131G</td>
<td>I48F, W105G</td>
<td>300-fold to DDATHF</td>
<td>DDATHF selection</td>
<td>23</td>
</tr>
<tr>
<td>Murine</td>
<td>S180</td>
<td>G890A</td>
<td>S297N</td>
<td>4-fold to MTX</td>
<td>MTX treatment in vivo</td>
<td>24</td>
</tr>
<tr>
<td>Murine</td>
<td>L1210-C8a</td>
<td>G133A</td>
<td>E45K</td>
<td>20-fold to MTX</td>
<td>Continuous exposure to MTX</td>
<td>25</td>
</tr>
</tbody>
</table>

MTX, methotrexate.
GW1843U89, (S)-2-[5-(((1,2-dihydro-3-methyl-1-oxobenzo-(f)quinazolin-9-yl)methyl)amino)-1-oxo-2-isoadolinyl]-glutaric acid.
DDATHF, 5, 10-dideazatetrahydrofolate.

Table 2 Clinical characteristics of the high-grade osteosarcoma samples

<table>
<thead>
<tr>
<th>Clinical characteristics</th>
<th>Without sequence alterations</th>
<th>With altered exon 2 and/or exon 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>139</td>
<td>23</td>
</tr>
<tr>
<td>Mean age</td>
<td>22.1</td>
<td>27.6</td>
</tr>
<tr>
<td>Sex</td>
<td>Male–66, female–61, unknown–12</td>
<td>Male–10, female–12, unknown–1</td>
</tr>
<tr>
<td>Primary site</td>
<td>Distal femur–52 (37.4%)</td>
<td>Distal femur–8 (34.8%)</td>
</tr>
<tr>
<td>Osteosarcoma</td>
<td>Proximal humerus–11 (7.9%)</td>
<td>Proximal humerus–5 (21.7%)</td>
</tr>
<tr>
<td>Chondroblastoma–25 (18.0%)</td>
<td>Proximal tibia–30 (21.6%)</td>
<td></td>
</tr>
<tr>
<td>Mixed–5 (3.6%)</td>
<td>Proximal femur/pelvis–12 (8.6%)</td>
<td>Proximal femur/pelvis–4 (17.4%)</td>
</tr>
<tr>
<td>Giant cell–4 (2.9%)</td>
<td>Unknown/other–34 (24.5%)</td>
<td>Unknown/other–1 (4.4%)</td>
</tr>
<tr>
<td>Pleomorphic–1 (0.7%)</td>
<td>Unknown/other–44 (31.6%)</td>
<td>Unknown/other–3 (12.9%)</td>
</tr>
<tr>
<td>Telangiectatic–5 (3.6%)</td>
<td>MFH–3 (2.2%), fibroblastic–5 (3.6%)</td>
<td>MFH–1 (4.4%), fibroblastic–1 (4.4%)</td>
</tr>
<tr>
<td>Metastases at diagnosis</td>
<td>No–89</td>
<td>No–12</td>
</tr>
<tr>
<td>Huvos grade</td>
<td>Yes–13</td>
<td>Yes–8</td>
</tr>
<tr>
<td>Status</td>
<td>NED–45 (NED–56.2%)</td>
<td>NED–10 (NED–58.8%)</td>
</tr>
<tr>
<td>Dead, WD–35 (Dead, WD–43.8%)</td>
<td>Dead, WD–7 (Dead, WD–41.8%)</td>
<td></td>
</tr>
<tr>
<td>Dead, WD–35 (Dead, WD–43.8%)</td>
<td>Dead, WD–7 (Dead, WD–41.8%)</td>
<td></td>
</tr>
</tbody>
</table>

MFH, malignant fibrous histiocytoma; NED, no evidence of disease; WD, with disease.

osteosarcoma samples at diagnosis have decreased RFC expression (11).

Recently, several mutations and polymorphisms of the RFC gene have been identified in cell lines that result in antifolate resistance. A polymorphism has been reported at amino acid 27 of the human RFC (12, 13), which will be discussed further in the “Discussion.” Several mutations have been reported in the RFC gene in antifolate-selected cell lines (Table 1; Refs. 14–25). Clusters of point mutations were observed in human and murine cell lines (21, 26), which might imply the
functional importance of those residues to the carrier. Therefore, a project to screen osteosarcoma patient samples for potential mutations and polymorphisms in the RFC gene was initiated. Initial screening was done with a DNA SSCP assay (21), followed by sequencing of RFC in all samples with abnormal SSCP mobility.

MATERIALS AND METHODS

Patients. Osteosarcoma tumor samples (n = 162) were collected from patients who had surgery at Memorial Sloan-Kettering Cancer Center and from patients enrolled on a Children’s Oncology Group biology study (P9851) between 1992 and 2000. All samples had a histologically confirmed pathological diagnosis of high-grade osteosarcoma. All patients provided written informed consent for tissue procurement and conduct of the biology study. Tumors were procured in accordance with a biology study approved by the Memorial Hospital Institutional Review Board and the institutional review boards of Children’s Oncology Group institutions that procured tissue for this study. One hundred and six samples were from Memorial Sloan-Kettering Cancer Center. Patients from whom these samples came were treated on or according to the pediatric Intergroup Phase III clinical trial (CCG #7921) described previously (1). The remaining samples were from patients treated as part of a Children’s Oncology Group therapeutic protocol, P9754, which was a series of pilot studies that explored the feasibility of doxorubicin dose intensification, ifosfamide dose intensification, or both. All of these protocols include multiple courses of high-dose methotrexate therapy. Histological necrosis after induction chemotherapy was determined according to the Huvos grading system as described previously (27).

DNA SSCP Assay. Genomic DNA was prepared from the osteosarcoma patient samples using a QIAamp DNA Tissue Kit (Qiagen, Inc., Valencia, CA) according to the manufacturer’s instructions. PCR-based DNA SSCP analysis spanning the entire coding region of the human RFC gene was done to screen for mutations or polymorphisms. The method was based on that described previously (21), with some modifications in primers and PCR conditions. Ten pairs of oligonucleotide primers were used. Five primer pairs had been described previously (21). The five additional primer pairs used were as follows: RFC460 (5′-ggagacctcaagggcatgctg-3′), RFC506 (5′-ttaggagaatagccagtgcg-3′), RFC680 (5′-ggtctggcccctctctggag-3′), RFC750 (5′-gcacgcccagggctc-3′), RFC839 (5′-gcctgatctgcggaggc-3′), RFC950 (5′-ccatgacgacaagcatg-3′), RFC1550 (5′-ggaagctttctctggagt-3′), RFC1600 (5′-gagacgctcaggtgcttcg-3′), RFC1650 (5′-gcagagacagacgcatctg-3′), RFC1800 (5′-gacacgacagctggagacg-3′), and RFC1900 (5′-gaaaggtacacagggc-3′). Genomic DNA was amplified with 2.5 units of Taq DNA polymerase (Invitrogen, Carlsbad, CA) in a 10-μl reaction volume containing 10 pmol of each primer, 0.2 mM deoxynucleotide triphosphate, and 2 μCi of [α-32P]dATP in the manufacturer’s reaction buffer. Samples were denatured at 94°C for 2 min, followed by 32 cycles at 94°C for 45 s, an annealing step for 45 s, and 72°C for 1 min, with a final extension at 72°C for 10 min. The annealing temperature was 52°C for exon 2 and 65°C for the remainder of the reactions. Stop solution (95% formamide, 20 mM EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol) was added to terminate the reactions. PCR products were resolved on a 6% neutral polyacrylamide gel that contained 10% glycerol running at 6 W for 12 h at room temperature. Then the gels were dried and exposed to X-ray films at −80°C overnight.

Direct Sequencing. DNA samples with different mobility patterns from those in wild type (arbitrarily defined as the sequence in the CCRF-CEM leukemia cell line, which transports methotrexate normally) as determined by SSCP analysis were PCR-amplified with high-fidelity Pfu polymerase (Stratagene, La Jolla, CA) in a 50-μl reaction volume that contained 5% DMSO using the conditions described previously. The PCR products were purified using a QIAquick Gel Extraction Kit (Qiagen, Inc.) and then cloned into a plasmid using a PCR-Script kit (Stratagene). At least five independent clones were manually sequenced from each PCR product using a T7 Sequenase version 2.0 sequencing kit (Amersham, Piscataway, NJ) according to the manufacturer’s instructions. All PCR products were sequenced in forward and reverse to confirm the sequence. At least two samples with the same SSCP mobility patterns were sequenced to confirm each sequence variant. Direct PCR product sequencing was done with a Sequenase PCR product sequencing kit (Amersham) and the appropriate RFC primers to confirm results, if sufficient genomic DNA was available.

Statistical Analysis. The histological response, patient outcome, and presence of metastases at diagnosis in the osteosarcoma samples with RFC sequence variants were related to the remainder using Fisher’s exact test.
RESULTS

Table 2 shows the clinical characteristics of the high-grade osteosarcoma patients, including their mean age at diagnosis, gender, primary disease site, histological subtype, and chemotherapeutic response as determined by the Huvos grading system (27). The clinical features of these patients are typical of those with this disease. Genomic DNA from the 162 osteosarcoma tumor samples was PCR-amplified using 10 pairs of oligonucleotide primers that spanned the entire RFC coding region and subjected to SSCP analysis. Fig. 1 shows representative SSCP analyses for exon 2 of the RFC. The pattern observed in the CCRF-CEM cell line, which transports methotrexate normally, is arbitrarily designated as wild type. The results of the SSCP analyses are summarized in Table 3. SSCP analysis of exon 2 found that only 61 of 162 samples (37.6%) had the same pattern as that of the wild type (Table 3). Most samples that differed from this sequence had one of two variant patterns arbitrarily designated as A (49 samples) and B (37 samples). Sequencing identified the wild type as being A/G heterozygous at RFC cDNA position number 174 [based on the nucleotide numbering of Prasad et al. (28), GenBank accession number U15939], encoding amino acid His27 and Arg27; variant A was homozygous G (Arg27) at this position, and variant B was homozygous A (His27) at this position (Table 4). Five other variant patterns (designated C–G) also were observed in RFC exon 2 in 15 samples (Table 4). The nucleotide sequence alterations in RFC exon 2 associated with each variant and the corresponding amino acid changes are summarized in Table 4. Each of the RFC sequence alterations generally was observed in more than one sample. One sample had a sequence alteration that resulted in a frameshift (Table 4). Representative sequencing gels of RFC exon 2 in CCRF-CEM (wild type) and two osteosarcoma patient samples with alterations are shown in Fig. 2. All of these alterations appeared heterozygously with the wild-type sequence.

Eight different samples had altered SSCP patterns in exon 3, which were associated with nucleotide changes that altered the amino acid sequence. These included a G to T substitution at cDNA position 869, a C to T substitution at cDNA position 759, a T to C substitution at cDNA position 855, and a T to C substitution at cDNA position 966, resulting in G259W, T222I, M254T, and L291P amino acid substitutions, respectively. These alterations also appeared heterozygous with the wild-type sequence. Fifteen samples had alterations in exon 2, thus 23 samples (14.2%) had exon 2 or exon 3 alterations resulting in amino acid sequence changes other than the cDNA position number 174 variation. In addition, 18 samples had homozygous C at cDNA position 790 compared with heterozygous C/T in the wild type. This sequence variation does not change the amino acid sequence. In RFC exon 4 and exon 5, no variant patterns were found with SSCP analysis (Table 3). Variant SSCP patterns were observed in RFC exon 6, but none were associated with sequence alterations when sequenced.

The clinical characteristics of the 23 patients with RFC sequence alterations other than the variation at cDNA position number 174 are summarized in Table 1. No significant difference was observed in this group compared with the remainder in terms of gender, primary site, histological subtype, and metastases at diagnosis or status. The samples with RFC sequence alterations had significantly higher frequencies of inferior histological response (Huvos grade I or II) after preoperative chemotherapy compared with the remainder (P = 0.016). Comparing the histological response for each of the RFC sequence variants, including variants His27 and Arg27, with the overall group, only exon 3, G to T substitution at cDNA position 869 was significantly associated with a higher frequency of an inferior histological response (P = 0.032).

DISCUSSION

This is the first study to report the screening of osteosarcoma patient samples for sequence alterations in the RFC. RFC sequence alterations were frequent in these patient samples. A
polymorphism described previously at cDNA position number 174 was observed at comparable frequencies, as reported previously (12, 13). In this study, it appeared that this polymorphism did not have an impact on the patients’ response to preoperative chemotherapy, which included methotrexate. This is consistent with observations by Whetstine et al. (13), who demonstrated that this polymorphism does not alter transport properties with \(^{3}H\)methotrexate and \(^{3}H\)5-formyltetrahydrofolate as substrates. However, in combination with the 667C/T polymorphism in the methylenetetrahydrofolate reductase gene, the 174G/A polymorphism might influence the folate status and total homocysteine level in a healthy population (12). Also, it was suggested that the 174G/A polymorphism plays a role in the risk of neural tube defects in newborns, which is associated with hyperhomocysteinemia in pregnant women (29, 30). A recent study showed this 174G/A polymorphism is correlated with methotrexate plasma level and outcome in patients with acute lymphoblastic leukemia.\(^{4}\) Further investigations are needed to clarify the functional role of this polymorphism.

In addition, 23 of 162 (14.2%) patient samples had other exon 2 or exon 3 alterations that resulted in amino acid sequence changes. In this study, SSCP analysis, which can be associated with false-positive and false-negative results, was used to screen the osteosarcoma samples for RFC sequence alterations. False positives, such as the SSCP pattern variability in exon 6, which was not associated with a RFC sequence change, were eliminated by confirming all alterations by manual sequencing. Sequencing of all samples with the wild-type SSCP pattern was not done, therefore false-negatives might have occurred. The ability to detect single-base changes with SSCP rests on several factors, including size of fragments, gel temperature, and addition of glycerol (31, 32). With the fragment size and conditions used, based on prior studies (31, 32) we anticipate that the sensitivity of the RFC SSCP analysis to detect point mutations would be 89–99%. Furthermore, only the coding regions were analyzed by SSCP and sequencing in this study, which left the untranslated, promoter, and intronic regions still unknown (21). The primers used in DNA SSCP analysis are intron-exon boundary derived, and thus they may not detect sequence alterations at each end of the exons. Therefore, it is possible that RFC sequence alterations in the osteosarcoma patient samples are even more frequent than the 14.2% detected in this study.

Most RFC sequence alterations in the present study have not been reported. The sites of amino acid alterations in human RFC identified in prior studies and the present study are shown in Fig. 3 (14, 18–21, 28, 33). Only one of the sites (amino acid position number 46) identified in this study has been reported as mutated in prior studies that used cell lines (17). In one prior study (21), the G (cDNA position 231) was substituted to a T, resulting in a serine to isoleucine substitution at amino acid 46, rather than the G to A substitution (Fig. 2B) in the present study, which changed the serine to an asparagine. The lack of concordance of these results with those observed in cell lines is perhaps not surprising. In antifolate-selected cell lines, mutations in dihydrofolate reductase are a mechanism of resistance (6, 34). In leukemia patients, mutations in dihydrofolate reductase have not been reported (6, 35), showing that cell lines do not always indicate what occurs in patient samples. The lack of dihydrofolate reductase mutations in patient samples and the frequent observation of RFC sequence alterations is interesting to note and might reflect differences in redundancy of enzyme functions, which might have implications for designing therapeutic strategies to overcome the relevant mechanisms of methotrexate resistance.

All of the sequence alterations appeared heterozygous. The possibility that the wild-type RFC sequence was from normal stromal cell DNA that contaminated the tumor sample cannot be ruled out. Lack of RNA from these tumor samples precluded investigation of the expression of the wild-type and sequence-
altered RFC in these tumor samples. Silencing of RFC expression by promoter methylation has been reported in cell lines (36) and will be investigated in future studies. It is possible that the wild-type RFC allele is not expressed in these tumor samples.

This study continues to suggest that impaired transport is the major mechanism of methotrexate resistance in osteosarcoma, along with previous studies by this laboratory (11). Responses to conventional-dose methotrexate in osteosarcoma are fairly limited compared with other malignancies such as acute lymphocytic leukemia, in which methotrexate is used routinely in conventional doses (6, 7). The improved efficacy of high-dose methotrexate in osteosarcoma may be because it overcomes this transport defect by achieving high intracellular levels of drugs that move into cells by passive diffusion or through a putative low affinity route (10, 37). These intracellular levels of methotrexate may be effective, providing folylpolyglutamate synthetase is intact. Thus, the efficacy of high-dose methotrexate would be retained even when the RFC is impaired in terms of quantity or quality. Approximately half of osteosarcoma samples have RFC sequence alterations (11). An additional percentage of the samples have RFC sequence alterations, some of which are likely to be functional and associated with methotrexate resistance. Although most current protocols for the treatment of osteosarcoma include other agents (1–5), all patients in this study received multiple courses of methotrexate, as described in “Materials and Methods.” In this study, RFC sequence alterations were associated with an inferior histological response (Huvos grade I or II) after preoperative chemotherapy, as was shown previously for decreased RFC expression (11). This suggests that the RFC sequence alterations might be a prognostic factor. The presence of multiple RFC sequence alterations, with potentially different clinical implications of each, will require larger studies to establish the prognostic value of each of the alterations.

All of the RFC sequence alterations identified in the present study occurred in exons 2 and 3. This may have implications for subsequent studies that screen patient samples for RFC sequence alterations. Some of the mutated residues (Fig. 3) found in this study are highly conserved (residue 21, 46, 254, and 291) or semiconserved (residue 7) in different species (38), which suggests that they are potentially important to the function of this carrier. Alterations at cDNA position number 231 (amino acid 46), as observed in this study, have been observed both in murine (17) and human (21) antifolate-resistant cell lines, as mentioned. Mutations in the 5′ region of the RFC affect protein localization and stability (22), which can be postulated as the effect of the exon 2 RFC sequence alterations observed in this study. Limited reported studies are available to elucidate the potential functional effects of the observed exon 3 RFC sequence alterations.

Studies are under way to identify the functional effects of each of the RFC sequence alterations through transfection of the altered cDNA into a RFC-null MCF-7 cell line (39). Expression vectors containing full-length RFC cDNA with each of the sequence alterations have already been constructed. Completion of these studies has been technically problematic, and therefore further results are not available to date.

At present, it is not clear if any of these sequence alterations, other than one, are germ-line or tumor specific. Sequencing of the RFC in normal tissue from the patients whose tumors have sequence alterations would clarify this issue. This cannot be accomplished from the cohort of patients included in this report because obtaining normal tissue and/or peripheral blood was not a component of the existing institutional review board-approved osteosarcoma biology study. An effort is under way to collect peripheral blood samples from osteosarcoma patients providing tumor tissue at this time to address this question in the future. It is likely that exposure to high-dose methotrexate plays a role in RFC mutagenesis in osteosarcoma tumor cells, and therefore one may postulate that many of the alterations are likely to be tumor-specific mutations. It must be acknowledged that these RFC sequence alterations only occur in a small percentage of patients, and silencing of the wild-type allele may not be present in all tumor samples because all of the sequence variants observed in this study are heterozygous, and therefore their overall contribution to methotrexate resistance is unclear. Additional studies also will be needed to clarify whether the RFC sequence alterations have a prognostic implication in osteosarcoma, and, if so, whether or not this is through induction of methotrexate resistance.

REFERENCES

Fig. 3 Predicted secondary structure of the human RFC as described previously, based on hydropathy analysis and membrane topology studies (28, 33), with mutated amino acid residues reported previously in solid circles (14, 18–21), and those identified in the present study highlighted with arrows.
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