Polymorphisms and Methylation of the Reduced Folate Carrier in Osteosarcoma

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Abstract High-dose methotrexate is a standard component in the treatment of osteogenic sarcoma. Impaired methotrexate uptake associated with decreased reduced folate carrier expression is a common mechanism of methotrexate resistance in osteogenic sarcoma samples. We investigated whether promoter methylation and

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polymorphisms in the 3' untranslated region are involved in regulating reduced folate carrier expression. In a cohort of 66 osteogenic sarcoma specimens, quantitative methylation-specific polymerase chain reaction and single-strand conformation polymorphism were performed. We found detectable levels of promoter methylation in 84.3% of samples. When related to the reduced folate carrier mRNA levels, a trend was observed that reduced folate carrier expression is lower in samples (median, 0.7) with greater than 10% DNA methylation as compared with those (median, 2.3) with less than 10% DNA methylation. The heterozygous polymorphisms of 2582 T/G and 2617C/T in the 3' untranslated region showed reduced folate carrier expression (median, 0.9) as compared with the wild-type 2582T and 2617C (median, 4.2). The data suggest promoter methylation and polymorphisms in the 3' untranslated region of the reduced folate carrier may be involved in its transcriptional regulation in osteogenic sarcoma. Further study is required to confirm this finding.

Introduction

Osteogenic sarcoma is the most common primary bone malignancy in children and young adults [12]. High-dose methotrexate (MTX) with leucovorin rescue is a major component of therapy in most current protocols for the treatment of osteogenic sarcoma [13]. However, responses to conventional-dose MTX in osteogenic sarcoma are fairly limited compared with other malignancies such as acute lymphoblastic leukemia [6], in which MTX is used routinely in conventional doses, suggesting an intrinsic resistance. In general, the mechanisms of MTX resistance include [30]: (1) impaired drug transport; (2) reduced drug accumulation because of decreased MTX

polyglutamylation, increased drug hydrolysis, or increased drug efflux; (3) alterations in the structure or expression of the target enzyme dihydrofolate reductase (DHFR); and (4) some novel mechanisms proposed recently [1].

MTX is delivered into cells predominantly through the reduced folate carrier (RFC) [11]. To generate sufficient intracellular MTX, RFC transport of MTX is critical to its efficacy. Since the human RFC cDNA was cloned [15, 17, 23, 25], intensive efforts have been made to explore its clinical relevance in different tumor models. In osteogenic sarcoma, previous studies suggest the basis of intrinsic MTX resistance is probably the result of impaired drug uptake. More than half of osteogenic sarcoma samples at diagnosis have decreased RFC expression in association with inferior response to preoperative chemotherapy [8]. Therefore, RFC expression may be a prognostic factor for patients with osteogenic sarcoma. However, although the role of the RFC in MTX resistance is well established in the literature [6, 11, 30], it was not directly manifested in comprehensive cDNA profiling studies by comparing good versus poor responders to chemotherapy in osteogenic sarcoma [10, 14]. The RFC is ubiquitously expressed in normal tissues [21]; its regulatory mechanisms are, however, not clearly understood. Transcription of the RFC starts from at least three distinct promoters (designated A, B, and C) and is complicated by multiple 5'-noncoding exons resulting from alternative splicing [5, 19, 21, 22, 24, 29]. The RFC promoter B appeared most potent in activity and was predominantly used in tumor cells [19, 22].

DNA methylation plays an important role in embryonic development and gene imprinting [2]. In cancer cells, DNA methylation at the promoter region is often involved in gene silencing or downregulation, particularly for some tumor-suppressor genes [9]. Recently, one study reported an approximately 1400-bp region, including RFC promoter B and A, identified as a CpG island. Promoter methylation in this CpG island was the underlying mechanism for the lack of RFC expression in a breast cancer cell line, MDA-MB-231, associated with resistance to MTX. Aberrant methylation in the promoter region was reportedly the potential mechanism of MTX resistance in primary central nervous system lymphomas [4]. In addition, polymorphisms of the 3' untranslated region (3'UTR) are also involved in the transcriptional and posttranscriptional regulation in some genes [3]. A polymorphism in the 3'UTR of the DHFR gene was suggested as having an impact on its mRNA levels. Two polymorphisms in the 3'UTR of the RFC are described [23, 25] in the single nucleotide polymorphism database of the National Center for Biotechnology Information. The function of these polymorphisms is unknown at present.

Further information on RFC regulation in osteogenic sarcoma is of importance for understanding the MTX

resistance in this disease. In this study, we sought to address two specific questions: (1) Is promoter methylation associated with the RFC downregulation frequently observed in osteogenic sarcoma samples? (2) Are there sequence polymorphisms in the 3'UTR of the RFC, and is there a difference in RFC mRNA expression among groups of osteogenic sarcoma specimens with these polymorphisms?

Materials and Methods

Our key study variables were the extent of promoter methylation and polymorphisms in the 3'UTR in the RFC. To relate these variables to RFC expression to determine if they were involved in the gene's transcriptional regulation we obtained osteogenic sarcoma tumor samples ($n = 66$) from patients treated at Memorial Sloan-Kettering Cancer Center (MSKCC) between 1996 and 2001. Thirty-four of patients were male and 32 were female. The average age was 22.5 years (range, 5–77 years). The location of the primary lesion was the distal femur in 37 patients, proximal tibia in 10 patients, and proximal humerus in five patients. All samples were confirmed to have a pathologic diagnosis of high-grade osteogenic sarcoma. The treatments of all patients included multiple courses of high-dose MTX. Thirty-one specimens were obtained from definitive surgery, 13 were from biopsy, and 22 were relapsed or metastatic lesions. Genomic DNA from these samples was extracted using a QIAamp DNeasy Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Adequate and sufficient genomic DNA was obtained from 51 samples for the promoter methylation study, and all 66 samples were subjected to polymorphism analysis, because a much smaller amount of genomic DNA was required for this analysis. For all 66 osteogenic sarcoma samples, the RFC mRNA expression levels ranged from 0.02 to 229.1 as measured by quantitative real-time reverse transcriptase-polymerase chain reaction in the previous study [18] (relative to that of the MTX transport normal leukemia cell line, CCRF-CEM). All patients provided written informed consent for tissue procurement and the conduct of this study, which was performed in accordance with the Memorial Hospital Institutional Review Board.

To measure the extent of RFC promoter methylation, a quantitative real-time methylation-specific polymerase chain reaction (MSP) was developed based on the methodology by Usadel et al. [20] and was validated in a panel of standard cell lines by direct bisulfite genomic sequencing. Bisulfite treatment was performed as described previously [26]. Briefly, 2 μ g of genomic DNA was denatured in 0.3 M NaOH for 15 minutes at 37°C. Samples were incubated for 16 hours at 55°C with freshly prepared

3 M sodium bisulfite (Sigma, St Louis, MO) (pH 5.0) containing 10 mM hydroquinone (Sigma). DNA was recovered with a DNA Clean-up Wizard Kit (Promega, Madison, WI) and was treated with 0.3 M NaOH for 15 minutes. Converted DNA was precipitated with 7 M ammonium acetate (pH 7.0) and cold ethanol. After washing with 70% ethanol, DNA was reconstituted in 30 μ L of water. Primers and fluorescent probes used in quantitative real-time MSP based on the methods described by Usadel et al. [20] were designed as follows: forward: 5'-TTG TCG TAG CGT TCG GTT AC; reverse: 5'-AAA CTA CAA CGC CCA CAA AA; and probe: 5'-Fam-TCG CGG GAC GGA TTC GTT TA-3'. The RFC basal promoter B was flanked by the polymerase chain reaction primers. Fluorescent real-time polymerase chain reaction was performed in a reaction volume of 50 μ L using components of a Taqman PCR Buffer A Pack (PE Biosystems, Branchburg, NJ). Each reaction used 3 μ L of treated genomic DNA including 1x Taqman buffer A with 600 nM each primer, 200 nM of probe, 200 μ M of dNTP mix, and 5.5 mM MgCl₂. Thermal cycling was carried out on a Bio-Rad iCycler (Hercules, CA) with a denaturation step of 95°C for 10 minutes, 50 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Genomic DNA treated with SssI methyl transferase (New England Biolab, Beverly, MA) was used as a positive control for methylated promoter. MYOD1 was used as internal control because of the lack of CpG sites on the primers and probe as described previously [20]. Multiple wells of nontemplate control were included on each polymerase chain reaction 96-well plate. All the samples were tested in triplicate for the RFC and MYOD1, respectively.

Polymerase chain reaction-based DNA single-strand conformation polymorphism (SSCP) was performed to screen polymorphisms of the 3'UTR of the RFC in osteogenic sarcoma samples. Four pairs of overlapping primers (3'UTR1–3'UTR4) were designed (Table 1). Genomic DNA was amplified with 2.5 units of Taq DNA polymerase (Invitrogen, Carlsbad, CA) in a 10- μ L reaction volume containing 10 pmol of each primer, 0.2 mM dNTP, and 2 μ Ci [α -³²P] dATP in the manufacturer's reaction buffer. Samples were denatured at 94°C for 2 minutes followed by

32 cycles at 94°C for 45 seconds, an annealing step for 45 seconds, and 72°C for 1 minute with a final extension at 72°C for 10 minutes. (See Table 1 for annealing temperature for each fragment.) Stop solution (95% formamide, 10 mM NaOH, 0.25% bromophenol blue, and 0.25% xylene cyanol) was added to terminate the reactions. Polymerase chain reaction products were denatured and resolved onto a 0.5x MDE gel (BioWhittaker Molecular Applications, Rockland, ME) running at a constant power of 6 W at room temperature for 12 hours. The gels were dried and exposed to radiographs at –80°C overnight as described previously [28]. Osteogenic sarcoma samples with a different SSCP mobility pattern from that observed in wild type (arbitrarily defined as that of CCRF-CEM) were polymerase chain reaction-amplified with Platinum Taq (Invitrogen, Carlsbad, CA). Polymerase chain reaction products were gel-purified using a Gel Extraction Kit (Qiagen, Valencia, CA) and subjected to manual sequencing in both directions using a T7 Sequenase version 2.0 sequencing kit (Amersham, Piscataway, NJ) according to the manufacturer's instructions. At least two samples with the same SSCP mobility pattern were sequenced to confirm a sequence variation.

The maximal chi square method was adapted to find the best cut point for methylation level according to mRNA expression of the RFC as previously described [20]. We also compared RFC mRNA expression according to the presence of polymorphism(s) in the 3'UTR either one at a time or comparing them as combinations using a Wilcoxon signed rank test.

Results

To address whether promoter methylation is the underlying mechanism of RFC downregulation frequently observed in osteogenic sarcoma samples, 51 samples were studied using the quantitative real-time MSP. Detectable levels of DNA methylation were observed in 43 (84.3%) samples. We found a cut point for methylation level of 10% best segregated the samples according to their RFC mRNA levels using the maximal chi square method. Fifteen

Table 1. Primers used in DNA-SSCP to screen polymorphisms in the 3'UTR

Fragments	cDNA position*	Sense primer	Antisense primer	Annealing T (°C)
3'UTR1	1843–2119	5'-gcgacggtgttcagaatgtg	5'-caccacacctctccagcaacaaa	58
3'UTR2	2068–2306	5'-atgctgtccctgcactgt	5'-cagctcagctacacctgag	56
3'UTR3	2245–2529	5'-acaggaggtgtgagccct	5'-acagacaggaccatggag	56
3'UTR4	2479–2788	5'-agctggttctctctctccca	5'-gggaagagtattcacatcacatca	55

* Numbering is based on Wong et al. [25], NCBI accession number U17920; SSCP = single-strand conformation polymorphism; UTR = untranslated region.

Fig. 1 Comparison of reduced folate carrier (RFC) mRNA expression in osteogenic sarcoma specimens with different levels of DNA methylation. (A) Samples ($n = 15$) with greater than 10% DNA methylation in the RFC promoter region, and (B) samples ($n = 36$) with less than 10% DNA methylation in the RFC promoter region. The middle lines indicate the median value (log) of RFC mRNA, and the filled boxes include samples with RFC mRNA levels ranging between 25th and 75th quartiles in each group. The lower and upper whiskers represent the minimum and maximum data values, respectively. The lines outside the whiskers represent outliers.

samples had more than 10% promoter methylation and 36 samples had less than 10% promoter methylation. We observed an insignificant ($p = 0.07$) trend for the RFC mRNA levels to be lower (median, 0.7) in samples ($n = 15$) with greater than 10% methylation as compared with that (median, 2.3) of samples ($n = 36$) with less than 10% methylation (Fig. 1).

To screen for polymorphisms in the 3'UTR of the RFC and to seek the possible relationship between the polymorphisms and mRNA levels of the RFC, DNA-SSCP was performed in all 66 osteogenic sarcoma samples. DNA-SSCP patterns corresponding to a novel polymorphism of 2100G insertion in fragment 3'UTR2 and two previously reported polymorphisms, 2582T/G and 2617C/T in fragment 3'UTR4, were identified and were confirmed by direct sequencing (Fig. 2). The latter two appeared in combination of 2582T with 2617C (wild type) as one allele or 2582G with 2617T (variant) as another allele in all samples studied (Table 2). When comparing mRNA level among samples according to these polymorphisms, that of the variant 2582 T/G, 2617 T/C heterozygote was lower

($p = 0.008$) than that of the wild-type 2582T, 2617C homozygote (Table 2). We observed insignificant differences in relation to RFC mRNA among the 2100G insertion variants in this group of samples (Table 2).

Discussion

We sought to address whether promoter methylation and the polymorphisms in the 3'UTR were involved in the regulation of the RFC expression in osteogenic sarcoma. In a previous study, we found decreased RFC expression in approximately half of the osteogenic sarcoma samples at the time of diagnosis, which was associated with an inferior response to preoperative chemotherapy [8]. The intrinsic resistance observed in osteogenic sarcoma is possibly the result of impaired RFC-mediated MTX uptake. Understanding the underlying mechanism of the RFC downregulation in osteogenic sarcoma is of importance to decipher the MTX resistance mechanisms in this disease.

The high frequency (84.3%) of DNA methylation in the RFC promoter region in osteogenic sarcoma samples revealed by quantitative MSP suggests it might be a mechanism of transcriptional regulation. However we observed only an insignificant trend for RFC expression to be lower in samples showing greater than 10% DNA methylation as compared with those showing less than 10% DNA methylation. Given the heterogeneity of clinical samples and possible contamination of stromal tissue, it is perhaps not a surprise that no difference was obtained between methylation level and mRNA expression of the RFC in this small group of osteogenic sarcoma samples. Promoter methylation was not only the underlying mechanism for RFC silencing in a breast cancer cell line, MBA-MD-231 [26], but was also identified as a potential mechanism of MTX resistance in a group sample with primary central nerve lymphoma as reported in a recent study [4]. High-dose MTX is a major component in most regimens for the treatment of patients with osteogenic sarcoma [13]. However, some tumors are resistant to MTX, probably through the RFC-mediated transport mechanisms [8, 28], and by overexpression of an alternative pathway for physiological folate uptake [27]. Laser-aided tumor microdissection, as well as expanded sample volume, might be helpful in a future study of osteogenic sarcoma and normal tissues to further clarify the role of promoter methylation in RFC expression regulation.

Polymorphisms of the 3'UTR are involved in the transcriptional and posttranscriptional regulation in some genes [3] such as DHFR [7], the target gene of MTX. With the fragment size and conditions used, based on prior studies [16, 28], we anticipate the sensitivity of the RFC SSCP analysis to detect point mutations would be 89% to 99%.

Fig. 2A–B Polymorphisms in the 3' untranslated region (UTR) of the reduced folate carrier (RFC). **(A)** DNA-single-strand conformation polymorphism (SSCP) assay revealed three major mobility patterns for each of the fragments shown, whereas that of the wild type (WT) is denoted with lines on the left. For 3'UTR2, wild-type, variant 1 (G 2100 insertion homozygote), and variant 2 (G 2100 insertion heterozygote) are pointed to by arrows. For 3'UTR4, wild-type (2582T, 2617C), variant 1 (2582G, 2617T), and variant 2 (2582 T/G, 2617 C/T heterozygote) are indicated by arrows. **(B)** Samples with distinct SSCP patterns were polymerase chain reaction-amplified and were directly sequenced. Nucleotides no 2086–2110 (U17920) of the RFC are shown and the position of a guanine insertion at cDNA 2100 is highlighted by arrows.

Table 2. Frequency of each polymorphism observed in the 3'UTR and the RFC mRNA expression in osteogenic sarcoma samples (n = 66)

Variants	Frequency	mRNA expression*
Wild type (3'UTR2)	36.4%	2.5
2100 G insertion homozygous	24.2%	2.9
2100 G insertion heterozygous	39.4%	1.6
Wild-type (3'UTR4) (2582T, 2617C)	22.7%	4.2
2582G, 2617T	40.9%	2.5
2582 T/G, 2617 C/T	36.4%	0.9

* The median value of mRNA expression in each group of osteogenic sarcoma samples; the mRNA level is relative to that of MTX transport normal cell line, CCRF-CEM [18]; UTR = untranslated region; RFC = reduced folate carrier; MTX = methotrexate.

The function of the polymorphisms observed in the 3'UTR of the RFC is unknown. The importance of the RFC expression being lower in the variant 2582 T/G, 2617 T/C heterozygote as compared with that of the wild-type 2582T, 2617C homozygote is not clear. Nevertheless, this study serves as the first step in understanding the transcriptional downregulation frequently observed in osteogenic sarcoma. Thus, promoter methylation and polymorphisms in the 3'UTR as regulatory mechanisms for RFC expression will need to be confirmed in future studies

and explored in the context of other transcriptional regulators.

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