mRNA Expression Levels of E2F Transcription Factors Correlate with Dihydrofolate Reductase, Reduced Folate Carrier, and Thymidylate Synthase mRNA Expression in Osteosarcoma[1]

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Abstract

Previous studies have shown that decreased expression of the reduced folate carrier (RFC) and increased expression of dihydrofolate reductase (DHFR) are associated with intrinsic and acquired methotrexate resistance, respectively, in osteosarcoma (OS). It has also been shown in colorectal cancer that E2F-1 expression correlates with thymidylate synthase (TS) and, to a lesser extent, DHFR expression. To begin to investigate the regulation of DHFR and RFC expression in OS samples, mRNA expression of E2F-1 and E2F-4 were measured in OS tumor samples and related to DHFR, RFC, and TS mRNA expression. Using fluorescent quantitative real-time PCR, 112 human OS patient samples were investigated for potential E2F-1/E2F-4:DHFR, E2F-1/E2F-4:RFC, and E2F-1/E2F-4:TS correlations. The expression ranges for each gene are as follows: DHFR, 0.02–33.13 (median = 0.20); RFC, 0.02–229.13 (median = 1.91); TS, 0.01–9.99 (median = 0.15); E2F-1, 0.05–69.07 (median = 0.52); and E2F-4, 0.24–52.35 (median = 1.45). Spearman correlation coefficients (r_s) for E2F-1:DHFR, E2F-1:RFC, E2F-1:TS, E2F-4:DHFR, E2F-4:RFC, and E2F-4:TS were calculated to be 0.53, 0.63, 0.60, 0.41, 0.58, and 0.33, respectively ($P < 0.001$). On the basis of this data, moderate correlations exist between E2F-1/E2F-4 and DHFR, RFC, and TS. These results suggest E2F-1/E2F-4 may play a role in the regulation of RFC expression, which has not been reported previously. The E2F transcription factors are also related to DHFR and TS expression in OS samples, suggesting a possible involvement in methotrexate resistance. Although E2F mRNA levels correlate with DHFR, RFC, and TS mRNA expression, additional experiments are necessary to determine the direct effects of these transcription factors and identify other proteins that may influence this relationship.

Introduction

Historically, conventional dose MTX[3] treatment has yielded unsatisfactory responses in OS patients, thus leading to the use of high-dose MTX, which is now standard in most current treatment protocols (1–5). Although this increased dosage has proven to be more effective, some OS tumors still do not respond and may be resistant to this chemotherapy. On the basis of preliminary data, it is thought that many OS tumors possess intrinsic MTX resistance through decreased expression of the RFC, resulting in impaired transport of MTX (6). In support of this hypothesis, previous work has shown that 65% ($n = 20$) of initial biopsy OS samples have decreased RFC gene expression using semiquantitative reverse transcription-PCR that was not secondary to gene deletions. Furthermore, 65% ($n = 26$) of those OS samples with an inferior degree of histological necrosis after induction chemotherapy (Huvos grade I–II) showed decreased RFC expression, suggesting this may be a determinant of patient outcome (6). Preliminary data also suggested DHFR overexpression may be an important mechanism of acquired MTX resistance in OS samples (6). Increased expression of DHFR, which plays a role in intracellular folate metabolism by regenerating tetrahydrofolate from dihydrofolate, leads to resistance, although increasing the amount of protein that needs to be competitively inhibited by MTX. In previous evaluation of OS samples, overexpression of DHFR was rare at the time of diagnosis (10% of initial biopsy samples) but was frequent after therapy (62% of metastatic or recurrent samples). This overexpression was not secondary to gene amplification as determined by Southern analysis (6). To better understand the basis of this resistance and the regulation of the expression of these genes, the mRNA expression levels of transcription factors that may be involved in their control were investigated.

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[3] The abbreviations used are: MTX, methotrexate; RFC, reduced folate carrier; DHFR, dihydrofolate reductase; OS, osteosarcoma; TS, thymidylate synthase.

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The expression of TS, a major target of 5-fluorouracil and its correlation with response of colorectal cancer patients to this therapy, has been extensively studied. TS expression at both the mRNA and protein level can serve as prognostic indicators in that increased expression is associated with poor outcome (7–9). A close association of TS with E2F-1 expression has been shown in colorectal cancer patient samples (10). The E2F family of transcription factors is known to be involved in the transcriptional regulation of several DNA synthesis enzymes and common chemotherapeutic targets (11). E2F-1 is the transcription factor most closely associated with TS expression (10, 11), whereas E2F-4 has been shown to be a regulator of DHFR expression (12). HT-1080 (fibrosarcoma) cell lines transfected to overexpress E2F-1 had increased expression of TS and, to a lesser degree, DHFR at both the protein and the mRNA levels (11). Possible correlations between E2F family members and RFC have not been investigated previously in patient samples, but given the relationship between E2F family members and DHFR and TS, as well as the similar cell cycle-dependent levels and requirements for these proteins, it would not be unexpected for E2F to play a role in the transcriptional regulation of RFC. In fact, SP1 sites, the binding sites for the E2F transcription factors, have been shown to be present in the RFC promoter (13).

This study was performed to investigate the possible relationship, if any, between the mRNA expression of E2F-1 and E2F-4 transcription factors and RFC, DHFR, and TS mRNA expression levels in OS patient samples. Real-time quantitative fluorescent reverse transcription-PCR was used to measure the mRNA expression levels of DHFR, TS, RFC, E2F-1, and E2F-4 in the 112 OS patient samples.

Materials and Methods

Sample Collection. All tumor samples were obtained at Memorial Hospital between November 1997 and June 2001 after obtaining written informed consent according to a biology study approved by the Memorial Hospital Institutional Review Board. All samples were confirmed to have a pathologic diagnosis of OS. A total of 112 samples was analyzed from 84 different patients.

RNA and DNA Preparation. Approximately 20 mg of fresh tumor tissue were frozen in Ultraspec Reagent (Biotecx, Houston, TX) and stored at −80°C. Total RNA was isolated using the Biotecx protocol with RNA resuspended in sterile, diethyl pyrocarbonate-treated water. To reduce the risk of genomic DNA contamination, mRNA was subsequently isolated from the total RNA suspension using a modified protocol from the Roche mRNA Isolation Kit (Indianapolis, IN). In short, a mixture of biotin-labeled oligo(dT) probe (30 pmol), hybridization buffer, and 300 μg of streptavidin magnetic particles was added to the total RNA suspension. After a 10-min 37°C incubation, the magnetic particles were separated using a magnet, and the supernatant was discarded. The remaining magnetic particles were washed twice (wash buffer), and the beads were resuspended in 20 μl of sterile, diethyl pyrocarbonate-treated water and incubated for 2 min at 70°C followed by magnetic separation. The supernatant (containing the mRNA) was immediately reverse transcribed using the Promega Reverse Transcription Kit (Madison, WI). After second strand synthesis, the cDNA was stored at −80°C.

Table 1 Real-time PCR primer and probe sequences

	Primer
DHFR (fwd)	5′-TAAACTGCATCGTCGCTGTGT-3′
DHFR (rev)	5′-AGGTTGTGGTCATTCTCTGGAAA-3′
DHFR (probe)	5′-6FAM-CCCGTTCTTGCCGATGCCCA-TAMRA-3′
RFC (fwd)	5′-CCTCCTGGTGTCAGCAAGCT-3′
RFC (rev)	5′-CCCGAGAGTCACTGGTTCACA-3′
RFC (probe)	5′-6FAM-CCGTCGCTTGGAAGACACTGCAAA-TAMRA-3′
TS (fwd)	5′-CTGTCCTGCCAGCTGTACCA-3′
TS (rev)	5′-GCGTAGCTGGCGATGTTGA-3′
TS (probe)	5′-6FAM-CCGAGGCCCATGTCTCCCGAT-TAMRA-3′
E2F-1 (fwd)	5′-CGGTGTCGTCGACCTGAACT-3′
E2F-1 (rev)	5′-AGGACGTTGGTGATGTCATAGATG-3′
E2F-1 (probe)	5′-6FAM-CTGCACCTTCAGCACCTCGGCA-TAMRA-3′
E2F-4 (fwd)	5′-CATCTGCTGTTTCTACACCTCCAC-3′
E2F-4 (rev)	5′-CTATTTGGACGTGAGGCTTCCT-3′
E2F-4 (probe)	5′-6FAM-AGCCTGCCCTAGCCCAGTCCCA-TAMRA-3′
β-Actin (fwd)	5′-TGAGCGCGGCTACAGCTT-3′
β-Actin (rev)	5′-TCCTTAATGTCACGCACGATTT-3′
β-Actin (probe)	5′-6FAM-ACCACCACGGCCGAGCGG-TAMRA-3′

Quantitative Real-Time PCR. The methodologies for the quantitative real-time PCR measurements have all been described previously. Standardization and validation of the assays have been described previously (10).[4] Briefly, 5 μl of cDNA were added to 20 μl of Master Mix containing: 1× Taqman Buffer A (PE Biosystems, Foster City, CA); 3.5 mM $MgCl_2$; 200 μM deoxynucleoside triphosphate; 0.025 units/μl Taq polymerase (AmpliTaq Gold; PE Biosystems); 900 nM each of forward primer and reverse primer; and 200 nM of probe. All probes were modified with a 6-carboxyfluorescein fluorophore at the 5′ end and a 6-carboxytetramethylrhodamine quencher at the 3′ end. All primers and probes (Table 1) were obtained from IDT (Coralville, IA) and validated for relative efficiency (14). All reactions were carried out using the Bio-Rad iCycler iQ (Bio-Rad, Hercules, CA) in 96-well plates. Cycling parameters were as follows: one 10-min hot start at 94°C; 45 cycles of 30-s denaturation at 94°C; 1 min of annealing at 55°C; 30-s extension at 72°C; and a final 5-min extension at 72°C. Each sample was run in triplicate for both the target gene and the housekeeping gene.

Real-Time PCR Data Analysis. To determine the relative quantitation of gene expression, the comparative C_T (threshold cycle) method was used (14). This method uses arithmetic formulas to determine relative quantitation. To normalize the samples for varying cDNA quantities, a housekeeping gene (*e.g.,* β-actin) is run concurrently with the gene of interest (*e.g.,* DHFR). For each sample, a threshold cycle (C_T) is determined for both the gene of interest and the housekeeping gene, which normalizes for cDNA quantity. Subtracting the C_T of the housekeeping gene from the C_T of the gene of interest yields the ΔC_T. For each run, identical cal-

[4] A. S. Levy, H. Sather, P. Steinherz, R. Sowers, M. La, J. Moscow, P. S. Gaynon, F. Uckun, J. R. Bertino, and R. Gorlick. Reduced folate carrier and dihydrofolate reductase expression correlate with outcome in acute lymphocytic leukemia: a children's cancer group study, submitted for publication.

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culations were made for a known positive control reference cell line. The CCRF-CEM leukemia line was used for DHFR and RFC, HOS human OS line for TS and E2F-4, and SkBr3 breast cancer line for E2F-1. The ΔC_T of the reference cell line was then subtracted from ΔC_T yielding the $\Delta\Delta C_T$, which was entered into the equation $2^{-\Delta\Delta CT}$, accounting for the exponential amplification of PCR. The reference cell line for each gene was arbitrarily assigned an expression of one. The expression of the gene of interest therefore represents the fold-difference expression relative to the reference cell line.

Statistical Analysis. The Spearman rank correlation coefficient (r_s) was used to determine correlative values between genes and groups of genes (*i.e.,* Huvos I–II *versus* Huvos III–IV and primary *versus* metastatic) because the observed data are not normally distributed. Because some patients contributed multiple samples (biopsy, definitive and/or relapse/recurrent at primary site and/or metastatic site), we used the bootstrap method to take the dependent measurements within patients into account. We resampled patients 1000 times, then 1000 Spearman rank correlation coefficients were calculated.

Results

mRNA was extracted from 112 OS samples (84 patients) and reverse transcribed yielding adequate cDNA to perform PCR amplification of DHFR, RFC, TS, and E2F-1 in triplicate. Because of limited quantities of mRNA, only 107 samples were available for the measurement of E2F-4. The clinical characteristics of the 84 OS patients are presented in Table 2. These characteristics are typical of this population of patients.

DHFR mRNA expression was present at detectable levels in 99% (111 of 112) of the samples with a median value of 0.2 (Table 3, Fig. 1). This median value represents an expression value that is 5-fold less than the positive control, the CCRF-CEM leukemia cell line. DHFR gene expression ranged from 0.02 to 33.13. Little variation was noted when the sample population was divided into primary site *versus* metastatic site (median = 0.20 and 0.19, respectively). The difference between median values for the poor responders (Huvos grade I, II; median = 0.28) *versus* the good responders (Huvos III, IV; median = 0.18) proved to be statistically insignificant ($P = 0.171$). Similar to previous work, 12% (11 of 90) of the nonmetastatic samples had high DHFR expression, defined as expression greater than the reference cell line CCRF-CEM. Increased DHFR expression did not correlate with either therapeutic response ($P = 0.706$) or specimen site ($P = 0.184$).

Normal RFC expression was evidenced in 65% (73 of 112) of the samples where normal expression was defined as expression equal to or greater than the CCRF-CEM cell line (Table 3, Fig. 1). Median value for the total sample pool was 1.91. The median RFC expression in metastatic site samples (median = 2.55) *versus* primary site samples (median = 1.77) differed by nearly the same factor as in Huvos I, II (median = 2.50) *versus* Huvos III, IV (median = 1.70). However, as with DHFR, the relationship was not statistically significant ($P = 0.177$ and $P = 0.129$, respectively). There was no significant correlation between RFC expression and sample site ($P = 0.281$) or Huvos grade ($P = 0.124$).

Table 2 Clinical characteristics of the OS patients (total sample number = 112, total number of patients = 84)

Age	
Range	3–77 years
Mean	23.4 years
Median	18 years
Gender	
Female	50
Male	62
Site	
Primary site	90
Metastatic site	22
Chemotherapeutic response	
Huvos I & II	43
Huvos III & IV	16
Unavailable/unapplicable	53
Histological subtype	
Chondroblastic	21
Giant cell rich	8
MFH type	2
Osteoblastic	32
Parosteal	3
Fibroblastic	4
Telangiectactic	5
Mixed or unknown subtype	37
Sample site	
Femur	52
Tibia	21
Humerus	11
Pelvis	7
Vertebral	4
Ulna	4
Skull	3
Other	10

The median levels of expression for TS, E2F-1, and E2F-4 in the total sample pool were 0.15, 0.52, and 1.45, respectively (Table 3, Fig. 1). When comparing median TS expression in the primary site samples *versus* the metastatic site samples, little difference was noted (0.02-fold). The good responders had a median TS expression 0.08 lower than the poor responders. E2F-1 and E2F-4 demonstrated similar expression levels in good and poor responders. In the primary *versus* metastatic site, E2F-1 median expression was essentially unchanged, whereas E2F-4 median expression was 0.21 higher in the metastatic site samples than in the primary site samples.

Spearman correlation values between E2F-1/E2F-4 and DHFR and RFC and TS are summarized in Table 4. With respect to the total sample pool, the tightest correlation appears to exist between E2F-1 and RFC ($r_s = 0.63$, $P < 0.001$) and the weakest correlation between E2F-4 and TS ($r_s = 0.33$, $P < 0.001$). Within the metastatic site sample pool, correlations for E2F-1:DHFR and E2F-4:TS were not significant with Ps > 0.150. The tight correlation that appears to exist between E2F-1 and RFC fluctuates slightly when the total sample pool is subdivided by either site of specimen or Huvos grade. No substantial fluctuations are noted in any of the Spearman correlation coefficients when the total sample population is divided into primary *versus* metastatic or Huvos I, II *versus* Huvos III, IV. DHFR and TS both appear to link

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Table 3 DHFR[a], RFC[a], TS[b], E2F-1[c], and E2F-4[b] expression values in OS patient samples

	DHFR		RFC		TS		E2F-1		E2F-4	
	Range	Median	Range	Median	Range	Median	Range	Median	Range	Median
Total samples (n = 112)	0.02–33.13	0.2	0.02–229.13	1.91	0.01–9.99	0.15	0.05–69.07	0.52	0.24–52.35 (n = 107)	1.45
Primary site (n = 90)	0.02–33.13	0.2	0.02–229.13	1.77	0.02–9.99	0.16	0.05–69.07	0.52	0.24–52.35 (n = 86)	1.61
Metastatic site (n = 22)	0.03–0.70	0.19	0.20–21.11	2.55	0.01–1.13	0.14	0.06–8.40	0.51	0.24–4.56 (n = 21)	1.82
Huvos I and II (n = 43)	0.03–22.78	0.28	0.14–125.37	2.5	0.02–9.99	0.2	0.08–69.07	0.67	0.24–18.64	1.78
Huvos III and IV (n = 26)	0.02–33.13	0.18	0.03–229.13	1.7	0.02–6.19	0.12	0.07–37.79	0.47	0.24–52.35	1.61

[a] Values are relative to the CCRF-CEM-positive control cell line.
[b] Values are relative to the HOS-positive control cell line.
[c] Values are relative to the SkBr3-positive control cell line.

more closely with E2F-1 than with E2F-4, whereas with RFC, the correlation values are comparable between E2F-1 and E2F-4.

Discussion

Resistance to MTX, a major component of OS therapy, can be secondary to decreased RFC expression, increased DHFR expression, and/or decreased FPGS expression. This study investigated the potential relationship between E2F transcription factors and DHFR and TS in OS patient samples. Significant correlations were observed, suggesting the potential involvement of the E2F pathway in antifolate resistance in OS. In addition, to the best of our knowledge, this is the first reported study to investigate the relationship between RFC and the E2F transcription factors in patient samples. Significant correlations were observed between E2F-1/E2F-4 and RFC mRNA expression, suggesting their involvement in transcriptional regulation and potentially MTX resistance in OS.

Briefly summarizing some of the information that is known about the E2F pathway, inactive E2F is complexed to the retinoblastoma gene product (pRB). Upon (hyper-) phosphorylation of pRB as part of the transition from the G_1 to S phase of the cell cycle, the E2F protein is released from the E2F-pRB complex and along with its binding partners (DP protein family) becomes available to transcriptionally activate a plethora of target genes, including many genes involved in DNA synthesis and chemotherapy response (*e.g.,* DHFR, TS, thymidine kinase, ribonucleotide reductase; Refs. 15–17). E2F is known to be involved in the transcriptional regulation of DHFR and TS (15). Both E2F-1 and E2F-4 were investigated because prior studies have suggested E2F-1 is involved in TS regulation (10), whereas E2F-4 is involved in DHFR regulation (12). The RFC promoter is known to have many features in common with the DHFR promoter such as the presence of Sp1 sites, but its regulation has been less extensively characterized (13). The majority of OS samples have nonfunctional pRB usually through large gene deletions or rearrangements (18–21). In SaOS-2, a human OS cell line known to have truncated and nonfunctional pRB, expression levels of DHFR and TS are 2–4-fold greater than the HT-1080 fibrosarcoma cell line with wild-type pRB, which would be expected secondary to increased levels of free E2F (22).

TS protein or mRNA levels have been shown to be a major determinant of response to 5-fluorouracil chemotherapy in colorectal adenocarcinoma (23–26). 5-Fluorouracil is not used in the treatment of OS. TS was primarily included in this study to allow comparability with prior studies. Consistent with prior observations in other tumor systems, E2F-1 correlated most strongly with TS expression. E2F-4 also correlated significantly with TS expression.

In contrast to what is observed in colorectal adenocarcinoma, levels of E2F and TS expression are not markedly higher in the pulmonary metastases as compared with the primary tumor in OS (10). This may reflect differences between the two tumor types. OS metastasizes to the lungs much earlier in its clinical course and has much more frequent alterations in the pRB pathway both of which may account for the difference. The relationships between E2F-1:DHFR and E2F-4:TS in metastatic site samples were statistically insignificant ($P = 0.337$ and $P = 0.202$). Data from the total sample population suggests that E2F-1 transcription factor could be an important regulator of DHFR expression, but whether this relationship is maintained through metastasis cannot be determined. DHFR amplification in metastatic samples has not been demonstrated in prior studies, but those studies have relied on Southern blots that are relatively insensitive. Studying metastatic OS samples for low-level DHFR amplification with PCR or dot blots should be considered in subsequent studies.

Also in contrast to prior studies, decreased RFC mRNA expression did not correlate with an inferior histological response to induction. The basis for the conflicting results is unknown, but prior studies have used relatively small numbers of OS samples. Additional prospective studies will need to be performed to determine the potential prognostic value of RFC expression in OS. When comparing the poor responders (Huvos grade I and II) to good responders (Huvos grade III and IV), there is a decreased median expression of all genes assessed in this study. It may be postulated that increased DHFR expression in the OS samples results in MTX resistance and therefore inferior response, but this

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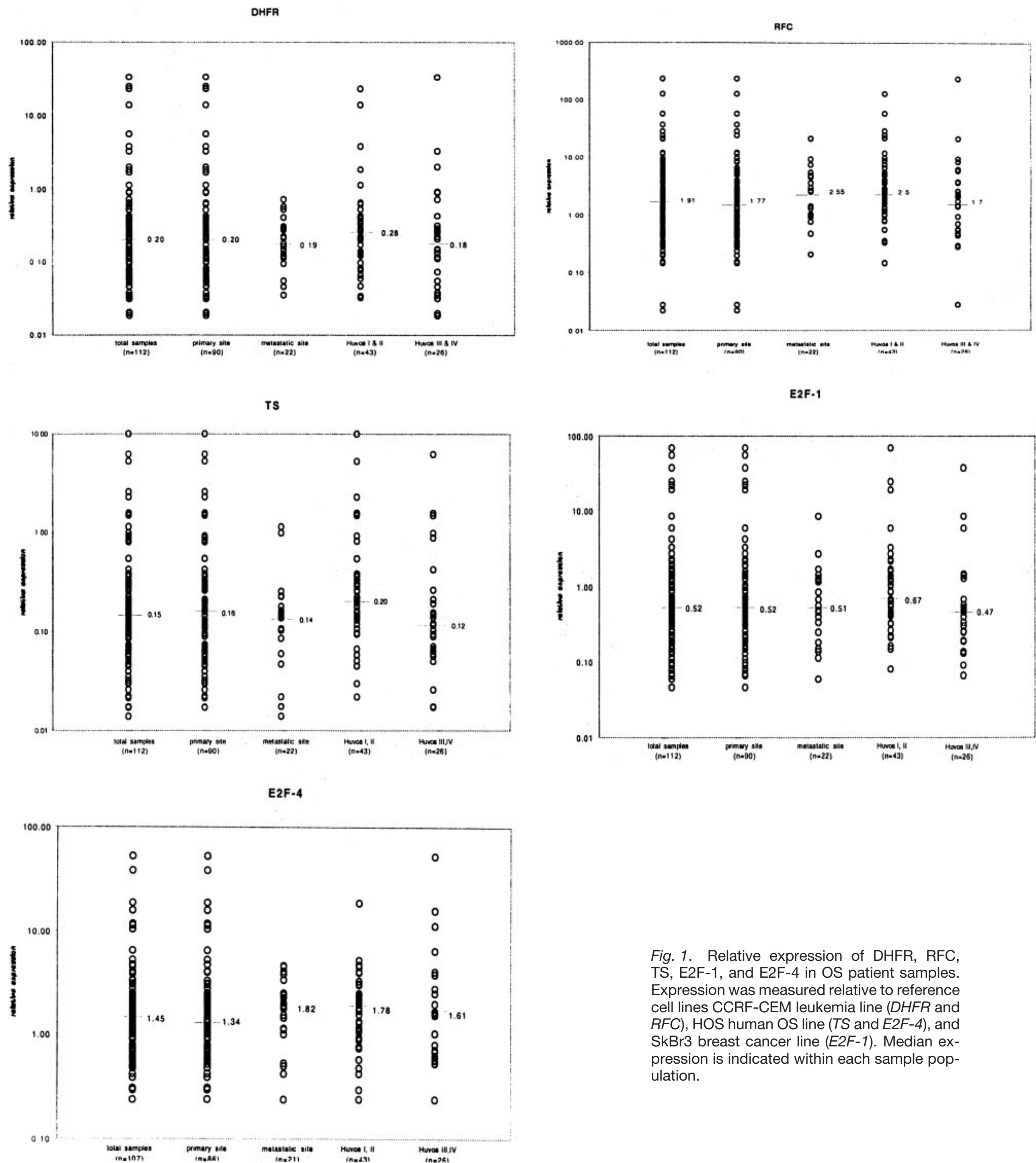

Fig. 1. Relative expression of DHFR, RFC, TS, E2F-1, and E2F-4 in OS patient samples. Expression was measured relative to reference cell lines CCRF-CEM leukemia line (*DHFR* and *RFC*), HOS human OS line (*TS* and *E2F-4*), and SkBr3 breast cancer line (*E2F-1*). Median expression is indicated within each sample population.

explanation could not be used for the RFC because, historically, decreased expression has been associated with MTX resistance. If decreased RFC expression is indeed an indicator of inferior histological response, the uniformly higher RFC mRNA expression of the poor responders in our study may be secondary to the tumor's cell cycle or proliferative state, which is being reflected in the expression of these S-phase genes. Additional studies will be necessary to determine which factors are primary determinants of histological response in OS and which are downstream events.

The association between E2F-1/E2F-4 and RFC should be regarded as preliminary yet informative. Identifying one of the transcriptional factors regulating RFC expression is impor-

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Table 4 Correlation (r_s) between E2F-1/-4 and DHFR, RFC, and TS in OS patients

	E2F-1:DHFR	E2F-1:RFC	E2F-1:TS	E2F-4:DHFR	E2F-4:RFC	E2F-4:TS
Total samples						
E2F-1 ($n = 112$);	0.53	0.63	0.60	0.41	0.58	0.33
E2F-4 ($n = 107$)	($P < 0.001$)	($P < 0.001$)	($P < 0.001$)	($P < 0.001$)	($P < 0.001$)	($P < 0.001$)
Primary site						
E2F-1 ($n = 90$);	0.61	0.61	0.71	0.40	0.55	0.34
E2F-4 ($n = 86$)	($P < 0.001$)	($P < 0.001$)	($P < 0.001$)	($P < 0.001$)	($P < 0.001$)	($P = 0.004$)
Metastatic site						
E2F-1 ($n = 22$);	N/S	0.74	0.60	0.35	0.59	N/S
E2F-4 ($n = 21$)	($P = 0.331$)	($P < 0.001$)	($P = 0.002$)	($P = 0.048$)	($P = 0.002$)	($P = 0.202$)
Huvos I & II	0.52	0.57	0.51	0.57	0.64	0.38
($n = 43$)	($P < 0.001$)	($P = 0.002$)	($P < 0.001$)	($P < 0.001$)	($P < 0.001$)	($P = 0.022$)
Huvos III & IV	0.47	0.67	0.80	0.35	0.63	N/S
($n = 26$)	($P = 0.014$)	($P = 0.006$)	($P < 0.001$)	($P = 0.070$)	($P < 0.001$)	($P = 0.160$)

tant, but it is likely to be only one of many factors that contribute to the regulation of this protein involved in MTX transport and resistance. For example, RFC promoter methylation has been shown to be associated with decreased RFC expression in some cell lines (27).

To understand these findings and their correlation with histological response to induction chemotherapy in OS, additional studies of the genes upstream of the E2F transcription factors should be performed. These studies should focus on pRB as well as other regulators of the pathway, including the cyclins, cyclin-dependent kinases, and cyclin-dependent kinase inhibitors. A simultaneous study of the various components of the pRB pathway along with the downstream target genes in OS tumor samples may allow the identification of which factors are independent determinants of chemotherapy response and subsequently patient outcome.

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mRNA Expression Levels of E2F Transcription Factors Correlate with Dihydrofolate Reductase, Reduced Folate Carrier, and Thymidylate Synthase mRNA Expression in Osteosarcoma [1]

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