[CANCER RESEARCH 60, 2365–2367, May 1, 2000]

Advances in Brief

Levels of E2F-1 Expression Are Higher in Lung Metastasis of Colon Cancer As Compared with Hepatic Metastasis and Correlate with Levels of Thymidylate Synthase[1]

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Abstract

We recently reported that forced overexpression of the transcription factor E2F-1 in human HT-1080 fibrosarcoma cells resulted in corresponding high levels of thymidylate synthase (TS) and resistance to 5-fluoropyrimidines (D. Banerjee *et al.*, Cancer Res., *58:* 4292–4296, 1998). Because colorectal metastasis to the lung has higher TS levels than liver metastasis and is less responsive to treatment with 5-fluorouracil (R. Gorlick *et al.*, J. Clin. Oncol., *16:* 1465–1469, 1998), it was, therefore, of interest to measure E2F-1 expression in these tumors. In contrast to marginally increased levels of dihydrofolate reductase and topoisomerase I in lung metastasis as compared with liver metastasis, lung tumors had a 5-fold increase in E2F-1 expression as compared with liver tumors, corresponding to the relative levels of TS in these metastases. These data indicate that there exists a close correlation between E2F-1 and TS levels and provide a rationale for targeting this transcription factor, *i.e.*, E2F-1, for the treatment of certain cancers.

Introduction

Colorectal cancer is a major cause of cancer mortality in the United States. Fluorouracil (5-FU) and fluorodeoxyuridine (FUDR) given systemically or via hepatic artery infusion remain standard treatment for advanced colorectal cancer (1). Metastatic disease to the lung and liver presents a significant problem in the successful management of advanced colorectal cancer. Although the primary tumors and, to a certain extent, the hepatic metastases remain responsive to fluoropyrimidine therapy, lung metastases are considerably less responsive to this therapy (2–4).

The levels of TS,[3] the target enzyme of the fluoropyrimidines, frequently correlates with, and may predict for, response to fluoropyrimidine-based therapies (5–9). High TS levels generally predict for lack of response, whereas lower levels are correlated with response. To understand the biochemical basis for the differential response of hepatic metastases and pulmonary metastases to fluoropyrimidine-based therapies, TS mRNA and protein levels were determined in colorectal tumor samples. Pulmonary metastases were found to express higher levels of TS mRNA and protein than hepatic metastases. High TS mRNA levels in some samples were associated with low-level gene amplification, but other samples with high TS expression were without any increase in gene copy number (10). TS and other proteins involved in DNA synthesis such as DHFR, TK, ribonucleotide reductase, and DNA polymerase α are coordinately regulated during the cell cycle (11, 12). These proteins are induced in mid-to-late G_1 phase of the cell cycle and participate in the orderly progression into the S phase. Regulation of cell cycle progression at the G_1 checkpoint seems to be largely dependent on the ability of pRb and related proteins to sequester and inhibit the activity of the E2F family of transcription factors (13, 14). Disruption of the pRb-E2F complex by inactivation of pRb leads to "free" E2F, which can then transactivate target genes such as *TS*. To examine the possibility that increased levels of E2F may lead to increased TS expression in the absence of gene amplification in the pulmonary metastases of colorectal cancer, we have initiated a systematic examination of factors that may influence levels of TS in these tumors. In the present study, we show that levels of E2F-1 expression in the pulmonary metastases are higher than in the hepatic metastases and correlate with the higher levels of TS in the former.

Materials and Methods

Preparation of Patient Samples. Eleven hepatic metastases and 7 pulmonary metastatic colorectal adenocarcinoma tumor samples were obtained from 17 patients. The tumors were obtained at the time of surgery with written informed consent of the patients. Diagnosis of disease was made at the Department of Pathology, Memorial Sloan-Kettering Cancer Center, using a portion of the tumor specimen. The remainder of the tumor sample was divided into three equal portions of at least 1 g each. The Ultraspec reagent (Biotexc Labs, Houston, TX) was added to one portion, and all of the three samples were flash frozen in liquid nitrogen until the time of DNA, RNA, and protein extraction. High-molecular-weight genomic DNA, total cellular RNA, and protein extracts were prepared as described previously (10). The paraffin-embedded sections were also used for immunohistochemical determination of protein expression.

QRT-PCR. The Taqman method used for QRT-PCR is based on the displacement of a fluorescent probe lying in between the region of interest in the particular cDNA, which is amplified and normalized to the displacement observed during amplification of a control gene such as *β-actin*. The ABI Prism 7700 Sequence detector (Perkin-Elmer, CT) was used for the QRT-PCR. The sequences of the various oligonucleotide primers used for amplification of DHFR, TS, E2F-1, Topo-1, and β-actin are: (*a*) for DHFR: probe, 5′-AGCCATGAATCACCCAGGCCATCTT-3′; forward primer, 5′-CTGGATAGTTGGTGGCAGTTCTG-3′; reverse primer, 5′-TGCATGATCCTTGTCACAAATAGTT-3′; (*b*) for TS: probe, 5′-AACATCGCCAGCTACGCCCTGC-3′; forward primer, 5-GGCCTCGGTGTGCCTTT-3′; reverse primer, 5′-GATGTGCGCAATCATGTACGT-3′; (*c*) for E2F-1: probe, 5′-CCTGCA-GAGCAGATGGTTATGGTGATCA-3′; forward primer, 5′-TGTCAGGACCTTCGTAGCATTG-3′; reverse primer, 5′-GGAGATCTGAAAGTT-CTCCGAAGA-3′; (*d*) for Topo-1: probe, 5′-CGTGTGGAGCACATCAATCTACACCCA-3′; forward primer, 5′-CTGTGGGCTGCTGCTCACT-3′; reverse primer,

Received 12/2/99; accepted 3/17/00.

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[1] Supported by Grants CA-61586 and CA-09512 from the National Cancer Institute (NIH).

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[3] The abbreviations used are: TS, thymidylate synthase; DHFR, dihydrofolate reductase; TK, thymidine kinase; pRb, retinoblastoma gene product; QRT-PCR, quantitative reverse transcription-PCR; Topo, topoisomerase.

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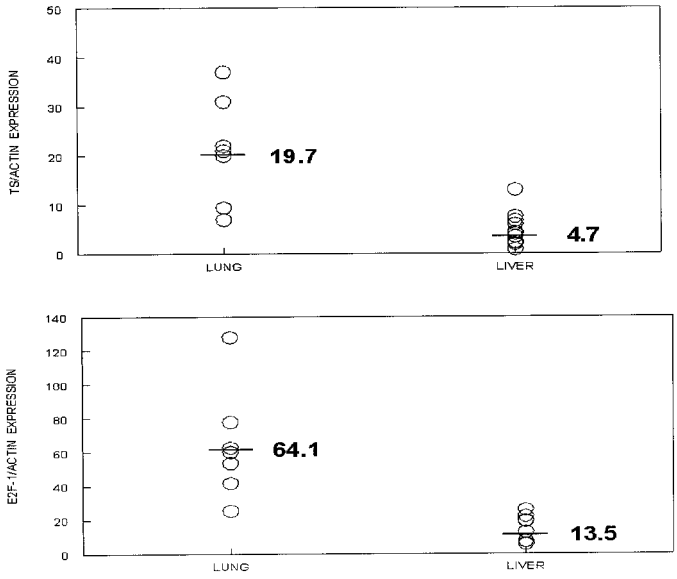

Fig. 1. Relative levels of TS and E2F-1 mRNA expression in lung and liver metastases of colon cancer. The *Y* axis scale is in relative units (specific mRNA expression/actin mRNA expression). The mean TS mRNA expression level in lung was 19.7, whereas that in the liver metastases group was 4.7 (*top*); the mean E2F-1 mRNA expression level was 64.1 in the lung metastases as compared with 13.5 in the liver metastases group (*bottom*).

5′-ACCACATATTCCTGACCATCCAA-3′; and (*e*) for β-actin: probe, 5′-ACC ACC ACG GCC GAG CGG-3′; forward primer, 5′-TGA GCG CGG CTA CAG CTT-3′; reverse primer, 5′-TCC TTA ATG TCA CGC ACG ATT T-3′.

Western Blot Analyses. For Western blot analyses, 100 μg of protein lysate was electrophoresed on a 10% polyacrylamide gel by SDS-PAGE and transferred to nitrocellulose. To determine equality of protein-loading, the nitrocellulose membrane was stained with Ponceau S stain. The membrane was then incubated overnight with blocking buffer and then with the E2F-1 antibody (obtained from Santa Cruz Biotech, Santa Cruz, CA) for 2 h. The excess primary antibody was washed off, and a secondary antibody (antirabbit IgG obtained from Santa Cruz Biotech) was applied to the membrane for 1 h. The secondary antibody binding was detected by the enhanced chemiluminescence (ECL, Amersham, Arlington Heights, IL) detection system as per manufacturer's instructions.

Immunohistochemical Analyses. Paraffin-embedded blocks were obtained from the Department of Pathology, Sloan-Kettering Memorial Hospital, and 4- to 5-μm sections were cut, deparaffinized, and rehydrated before further processing. Slides were pretreated by digestion with 0. 05% trypsin, and antigen retrieval was accomplished by microwaving the slide for 10 min at high power. Slides were incubated for 16 h at 4°C with the primary antibodies, were washed, and were incubated further with a biotinylated secondary antibody. Detection was carried out using peroxidase-conjugated streptavidin and incubation in 0.06% diaminobenzidine. The slides were counterstained in hematoxylin. Appropriate positive and negative controls were used for all of the antibodies.

Results and Discussion

Of the 18 tumor samples studied, 11 were hepatic metastases and 7 were pulmonary metastases of colorectal adenocarcinoma. The clinical details of the patients and TS expression in tumors was described earlier (10). TS expression was significantly higher in pulmonary metastases (mean TS:β-actin = 19.7) than in hepatic metastases (TS:β-actin = 4.7). The mean level of E2F-1:β-actin gene expression in pulmonary metastases was 64.1 as compared with 13.5 in the hepatic metastases, which correlated well with *TS* gene expression (Fig. 1). Although increased TS expression correlated with increased *TS* gene copy number (10), there were some tumor samples in which increased TS expression was observed in the absence of gene amplification. For example, pulmonary metastases samples L2, L4, and L5 had high levels of TS expression without *TS* gene amplification, and also had high levels of E2F-1 expression. The high levels of E2F-1 mRNA expression also correlated with increased protein levels, as measured by Western blot analyses (Fig. 2) and immunohistochemistry (not shown). Samples L3 and L7, which had high levels of TS expression attributable to increased gene copy number for TS, had moderate levels of E2F-1 protein expression. E2F-1 protein levels were undetectable in L6, although this sample had fairly high TS and E2F-1 mRNA expression levels. To determine whether expression of DNA synthetic genes in general was elevated in the pulmonary metastases, QRT-PCR was carried out for DHFR and Topo-I. The median value for DHFR:β-actin expression in hepatic metastases was 0.73 as compared with 1.39 in pulmonary metastases. For Topo-I expression, the median value for the lung metastases was 5.4, whereas that for the liver metastases was 3.5, indicating a 1.5-fold increase in mRNA levels (Table 1). There was a strong, statistically significant correlation ($P < 0.001$) between the levels of TS expression with the levels of E2F-1 expression (normalized to β-actin levels) with a correlation coefficient of 0.726, which suggested that E2F-1 is involved in the expression of TS (Fig. 3). We have shown recently (15) that forced expression of E2F-1 in HT-1080 cells (a human fibrosarcoma cell line) leads to a greater increase in levels of TS than either DHFR or TK levels, which is in agreement with the analyses pre-

Table 1 *Relative gene expression of TS, E2F-1, DHFR, and Topo-1 in lung (L) and liver (H) metastases*[a]

Sample	TS	E2F-1	DHFR	Topo-1
H1	3.4	19.3	0.89	1.2
H2[b]	7.5	25.8	5	8
H3	2.3	22	0.82	4
H4	2	5.6	1.86	3.5
H6	2.3	12.6	0.73	1.9
H7	4.1	12.5	0.34	2.5
H8	5.9	7.2	0.54	1.2
H9	13	5.5	0.27	6.6
H10	0.8	ND[c]	8.89	0.2
H11[b]	4.3	18.9	0.51	1.8
H12	6.7	5.4	0.56	7.9
Mean	4.7	13.5	0.73	3.5
L2	6.9	25.3	1.14	1.5
L3[b]	22	127.8	3.07	4.1
L4	20.7	62.5	3.1	3.8
L5	37	60	0.21	3.5
L6[b]	21	53.4	1.39	6.5
L7[b]	21	77.7	1.09	7.2
L8[b]	9.4	41.7	2.82	10.9
Mean	19.7	64.1	1.39	5.4

[a] Values are relative to β-actin levels.
[b] *TS* gene amplification present (10).
[c] ND, not done.

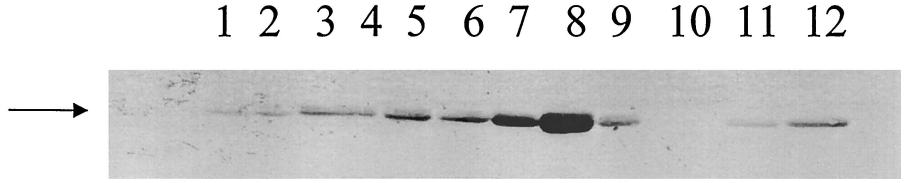

Fig. 2. E2F-1 protein levels as determined by Western blot analyses of lysates prepared from tumor samples of both lung (L) and hepatic (H) metastases. *Lanes 1–5*, samples H2, H5, H7, H8, and H12, respectively; *Lanes 6–12*, L2, L3, L4, L5, L6, L7, and L8, respectively.

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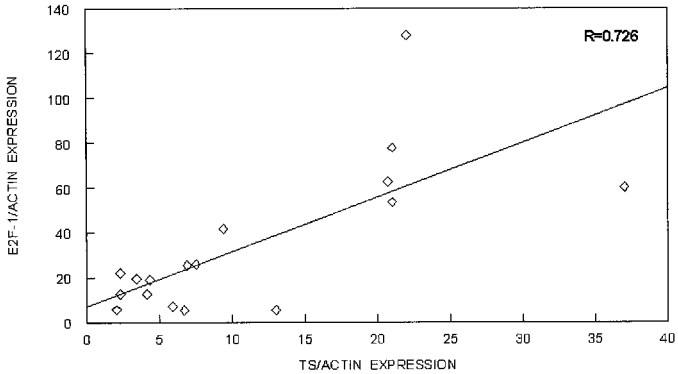

Fig. 3. Plot of E2F-1 levels *versus* TS levels indicates a correlation between the levels of E2F-1 and TS with a correlation coefficient of 0.726 ($P < 0.001$).

sented for the tumor samples. The present data suggest that increased expression of TS may be a consequence of *TS* gene amplification, or overexpression of E2F-1, or both in pulmonary metastases. We are currently investigating the reasons for the increased E2F-1 expression levels in the pulmonary metastases in comparison with liver metastases. Inasmuch as TS expression levels in primary colorectal carcinoma and the hepatic and pulmonary metastases are a reflection of the average E2F-1 expression levels in these tumor samples, this indicates that there is a trend toward increased E2F-1 expression levels in metastatic progression of colorectal carcinoma. The functional interaction between pRb and E2F regulates the G_1-to-S phase transition in the cell cycle. The ultimate hyperphosphorylation of pRb, either by the lack of pl6-induced inhibition of cyclinD1/cdk4 kinase activity or by the increase of such activity, leads to the disruption of the E2F pRb complex and, thus, releases the "molecular brake " on the G_1 checkpoint, which prompts cells to move into the S phase. This is accompanied by a concomitant increase in the levels of proteins required for DNA synthesis, such as DHFR, TK, TS, ribonucleotide reductase, and DNA Pol α. In normal cells, unscheduled expression of E2F-1 leads to rapid apoptosis, but tumors that have acquired several mutations especially, in the apoptotic pathway, *e.g.,* p53 and p14 ^{ARF}, will be further stimulated to grow in the presence of high levels of E2F-1 (16). It is of interest that, in malignant human bronchial epithelial cell lines, the levels of E2F-1 expression were increased and the index of E2F-1 $\times$ c-myc/p2l separated the malignant cells (all small-cell lung cancers) from the normal bronchial epithelial cells. This index was useful in separating seven malignant primary lung tumors from nine normal samples (17). The present study demonstrates that metastatic colorectal tumors in the lung are associated with elevated levels of TS and E2F-1. The finding that pulmonary metastases have even higher levels of E2F-1 and TS expression than liver metastases raises the issue of whether the progression of colorectal cancer (usually from liver to lung) is related to these increased levels of TS and E2F-1, or whether the soil, *i.e.,* the pulmonary tissue is contributing to the increased levels of TS and E2F-1. This can be addressed by examining levels of E2F-1 and TS in liver and pulmonary metastases obtained from the same patient. Targeted therapy, directed toward the elevated E2F-1 levels found in these and other tissues, such as tumor-selective transgene expression of a proapoptotic gene downstream of a promoter element that is responsive to E2F-1, may be worthwhile (18).

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