Increased Expression of Metallothionein Is Associated with Irinotecan Resistance in Gastric Cancer

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Abstract

To gain insight into clinically relevant mechanisms of irinotecan resistance, we undertook oligonucleotide microarray analyses on paired malignant effusion samples obtained from eight gastric cancer patients treated with weekly irinotecan. Pretreatment and posttreatment (48 h) effusion samples were obtained for each patient, and the change in expression profile was compared between clinical responders and nonresponders. When differences in the expression of genes were examined using SAM (Significance Analysis of Microarrays) software, five isoforms of the metallothionein family were identified to have significantly higher signal log ratios in five nonresponders, compared with three responders. Compared with control cells, metallothionein IX (MTIX)-transfected AGS cells showed a 1.4-fold higher irinotecan IC50 by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and tended to form more colonies. These findings collectively suggest that irinotecan-induced upregulation of metallothionein might be associated with irinotecan resistance in patients with gastric cancer, although it remains to be confirmed in a larger data set.

Introduction

Understanding the mechanisms of drug resistance could lead to a more rational basis for cytotoxic chemotherapy. Irinotecan, a camptothecin derivative, is a DNA topoisomerase I inhibitor that is active against a broad spectrum of solid tumors including gastric cancer tumors (1). Irinotecan treatment results in a collision between topoisomerase I cleavage complexes and DNA replication forks that produces irreversible double-strand breaks, ultimately leading to cell cycle arrest and death by modifying the expression of many genes (2). Limited information is available about how these genetic responses are differentially regulated between those who respond to irinotecan treatment and those who do not. To gain insight into the clinically relevant mechanisms of irinotecan resistance, we undertook DNA microarray analyses on paired gastric cancer effusion samples obtained before and after irinotecan treatment.

Materials and Methods

Patient Eligibility and Treatment. Metastatic gastric cancer patients with cytologically confirmed, malignant peritoneal or pleural effusion were eligible for the study. All of the patients signed an Institutional Review Board-approved informed consent form. Prior chemotherapy was allowed, and measurable lesion was not a prerequisite for the enrollment. Irinotecan (Campto; Aventis Pharma) was administered i.v. at 125 mg/m2 for 90 min once a week, four times, at 6-week intervals until there was evidence of disease progression. Response to irinotecan was evaluated every cycle (i.e., every 6 weeks), based on (a) computed tomography performed every cycle and (b) clinically detectable amount of effusion. Amounts of effusion was clinically assessed on days 1, 8, 15, and 22 of each cycle, based on the frequency of paracentesis/thoracentesis and diuretic consumption. Each patient was rated “improved,” “unchanged,” or “aggravated” for each of the two measures (i.e., computed tomography and clinical evaluation). To be classified as “responders,” patients had to be rated improved for one of the two measures without being assessed as aggravated for the other factor. Otherwise, patients were classified as “nonresponders.” Pretreatment and posttreatment (48 h) effusion samples were obtained for each patient, and the change in expression profile was compared between responders and nonresponders.

Sample Collection. Effusion samples were obtained before irinotecan treatment, along with those obtained for conventional cytological examination, and 48 h after the start of the day-1 irinotecan infusion for each patient. The samples were centrifuged within 10 min of collection at 3000 × g at 4°C for 5 min. The cell pellet was resuspended in TRI Reagent (Molecular Research Center Inc., Cincinnati, OH) and was subjected to mechanical homogenization. Total RNA extracted was treated with DNase I at 37°C for 30 min. RNA integrity was checked by agarose gel electrophoresis. The cell block section of diagnostic effusion sample was examined for the estimation of tumor cell percentage.

DNA Microarray Analysis and Semiquantitative Reverse Transcription-PCR. DNA microarray analysis was performed using 3–8 μg total RNA and HG-U133A oligonucleotide array containing 22,283 transcripts, according to the manufacturer’s recommendations (Affymetrix, Santa Clara, CA). Scanned data were processed using Affymetrix Microarray Analysis Suite (MAS) software version 5.0, with all of the parameters set at default values. To find genes with differential regulation, we performed Significance Analysis of Microarrays (SAM) with only 732 genes that had “present” (P) MAS detection calls in all of 16 samples (thus, genes that were present before treatment and were not present after treatment were excluded from the analysis). Specifically, MAS-generated, signal log ratio (SLR; 2SLR = fold change) values for these 732 genes of each patient sample pair were compared between responders and nonresponders, using various SAM parameters. Primer sequences of MTIX for semiquantitative reverse transcription-PCR were 5′-GGGGCTCTGGCCTTCTTTTTGAT-3′ (sense) and 5′-ATAGAAAAAAGAGTGAGAAACGCGG-3′ (antisense). Concentration of SN-38, an active metabolite of irinotecan, in ascites supernatant was measured by high-performance liquid chromatography as described previously (3).

MTT Assay and Colony-Forming Assay with MTIX Adenovirus Vectors. The metallothionein IIX (MTIX) fragment was PCR amplified with primers 5′-GGGGCTCTGGCCTTCTTTTTGAT-3′ (sense) and 5′-GGGGCCTTCTGGCCTTCTTTTTGAT-3′ (antisense), digested with NcoI and ClaI, and was cloned into the corresponding sites of pAChCMV-3.0 adenovirus shuttle vectors containing cytomegalovirus early promoter, to yield pAChCMV-3.0-MTIX expression cassette. pAChCMV-3.0-MTIX and empty pAChCMV-3.0 were cotransfected with adenovirus backbone vector pM17, to generate recombinant adenovirus Ad-MTIX and Ad-Null, respectively (4, 5). For 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and colony-forming assay, AGS cells (a human gastric adenocarcinoma cell line) were transfected for 48 h with either Ad-MTIX [10 multiplicities of infection (MOI)] or Ad-Null (10 MOI), and incubated under various concentrations of irinotecan for 72 and 24 h (followed by 12 days of incubation in drug-free media), respectively.
Eight patients (five males and three females) were enrolled on this study, from September 2002 through December 2003 (Table 1). All of the patients had cytologically confirmed, malignant peritoneal (n = 7) or pleural (n = 1) effusions. Two patients were chemotherapy-naive, and the other six patients had been treated previously with chemotherapy. Pretreatment samples were collected 1–4 days before the day-1 irinotecan treatment. The median percentage of cancer cells in the diagnostic effusion samples was assessed as 75% by light microscopy. When genomic DNAs extracted from all of the effusion samples (and from frozen endoscopic biopsy tissue of patients 1, 2, and 7) were subjected to direct sequencing for TP53 mutations in exon 8 (CGT to CAT), patient 2 showed a missense mutation at codon 273 (patient 2; endoscopic sample) suggested a missense mutation at codon 273 in exon 8 (CGT to CAT).

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First, we performed average linkage hierarchical clustering of all of 16 samples using the entire 22,283 probe sets (Fig. 1A) and a set of 732 genes that had present (P) MAS detection calls on all of 16 samples (Fig. 1B). Pre- and post-irinotecan samples from the same patient tended to be located more closely together than parallel samples from different patients, suggesting that treatment-related changes in gene expression profile were less prominent than individual variations in transcriptome profile. Two pleural effusion samples from patient 1 merged into a cluster distant from that of the other fourteen ascites samples (Fig. 1A).

We then looked at irinotecan-induced gene expression changes in relation to the clinical response of this particular subset of gastric cancer patients. Among the eight patients who participated in this study, three were classified as clinical responders and five as nonresponders to irinotecan treatment. Irinotecan-induced change in gene expression was compared between responders and nonresponders, using SAM software and Affymetrix MAS-generated SLR values for transcripts of each patient sample pair. As shown in Fig. 2A, eight positively significant probe sets (i.e., SLRnonresponder > SLRnonresponder) were identified on SAM (three nearest neighbors) at fold change of 2.0, median false significance rate of 12.5%, and δ 0.198. Included in seven genes represented by these eight probe sets were five isoforms of the metallothionein (MT) gene family. Two-tailed parametric P values for the comparison of SLR were 0.009, 0.034, 0.038, 0.044, and 0.038, for MT1F, MT1G, MT1X, MT1L, and MT1E, respectively. Semi-quantitative reverse transcription-PCR data for one of these isoforms, MT1X, were consistent with microarray results (Fig. 2B).

The other differentially expressed genes included GIP3 and GIP2, which are known as IFN-α-inducible genes.

The ascitic concentration of SN-38, an active metabolite of irinotecan, at 48 h posttreatment could be measured in a nonresponder and two responders. A nonresponder (patient 5) showed the SN-38 concentration of 2.1 ng/ml (patient 5) and 3.2 ng/ml (patient 8), respectively.

To evaluate whether MT1X overexpression alone could confer resistance to a gastric cancer cell line, we performed MTT assays and colony-forming assays using AGS cells transduced with either Ad-MT1X (10 MOI) or Ad-Null (10 MOI) for 48 h. Three independent MTT assays consistently demonstrated a modest increase in irinotecan IC50 after MT1X transfection, with 1.4-fold higher average IC50 in MT1X-transfected AGS cells than in control cells (P for t test = 0.0011; Fig. 3B). And MT1X-transfected AGS cells tended to form more colonies than did control cells after irinotecan exposure for 24 h, as shown in Fig. 3C.

Discussion

In this study, we tried to measure differential pharmacodynamic effects between responders and nonresponders, by monitoring irinotecan-induced gene expression change in readily available effusion samples.
samples, although free-floating cells may differ from primary tumor cells in drug response because of differences in microenvironment. Because this study focused on differential gene expression changes that were consistently found in malignant effusion across the various milieus, heterogeneous (i.e., 14 peritoneal and 2 pleural) effusion samples were analyzed together, which presumably might be related to the low number of genes having present (P) calls on all of the samples. Subtle or earlier (H11021 48 h) signal changes might have been undetected in the present analysis, given that we focused on relatively prominent changes in gene expression after a fixed time interval.

Although the majority (seven of eight) of samples had more than 50% of tumor cells, DNA microarray signals of the present study were derived from both tumor and normal cells. We assumed that the cellular composition of post-irinotecan effusions was similar to that of pre-irinotecan effusions and that the differential change in gene expression identified herein was not significantly affected by individual variation in tumor cell percentage change after irinotecan. Indeed, two patients (patients 5 and 8), whose posttreatment samples were tested for cellular composition, showed no difference in tumor cell percentage between pre- and posttreatment samples. We did not serially measure the effusion concentrations of irinotecan or its metabolites for all of the patients. In a mouse study, the area of the concentration-time curve (AUC) of SN-38, an active metabolite of irinotecan, was higher in ascites than in plasma and plasma SN-38 was rapidly distributed to and equilibrated with ascites (6). At 48 h after irinotecan administration, much time would have passed because SN-38 had reached elimination phase (7). Thus, the higher ascitic concentration of SN-38 in a nonresponder indicated that he had been exposed to more ascitic SN-38 than two responders had been, which could contradict a possible hypothesis that the differential expression signature identified herein might reflect a dose-response relationship (i.e., the lower SN-38 AUCs in nonresponders compared with responder samples).
sponders), rather than the individual pharmacodynamic characteristics.

Notably, it was the change in MT expression level, not the baseline expression level, that correlated with the clinical response of the study patients. Expression of MT, a highly inducible, ubiquitous protein, is primarily controlled at the level of transcription, and a great variety of substances and agents induce or repress MT transcription (8). Several lines of evidence suggest that MT is chemotherapy inducible (9), and its expression constitutes a protective mechanism that prevents the apoptosis induced by cisplatin and doxorubicin (10, 11), although the association between MT up-regulation and irinotecan resistance has not been previously reported. A role for p53 on the induction of MT in epithelial cells was suggested by published data (12), but TP53 mutation was detected in only one patient of the present study.

Taken together, irinotecan-induced change in MT expression correlated with clinical response in this subset of gastric cancer patients, and MT overexpression modestly increased resistance of AGS cells to irinotecan. These findings collectively suggest that irinotecan-induced up-regulation of MT may be associated with irinotecan resistance in patients with gastric cancer, although it needs to be confirmed in a larger data set.

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