Drg1 Expression in 131 Colorectal Liver Metastases: Correlation with Clinical Variables and Patient Outcomes

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Abstract

Purpose: The differentiation-related gene-1 (*Drg1*) is a recently identified gene down-regulated in malignancy and a putative suppressor of colorectal cancer metastases. Its expression is associated with improved survival in patients with prostate or breast cancer. Drg1 expression is also associated with resistance to irinotecan therapy in preclinical colorectal cancer models. The clinical evaluation of Drg1 in colorectal cancer has been limited. We performed this study to evaluate the role of Drg1 in a large cohort of patients with metastatic colorectal cancer who were irinotecan naïve.

Experimental Design: We examined Drg1 expression by immunohistochemistry in 131 patients with metastatic colorectal cancer enrolled in a clinical trial of adjuvant fluorouracil-based therapy from 1991 to 1995. We correlated expression of Drg1 to numerous clinical and tumor related variables and to patient outcomes, including a subset of patients who recurred and received irinotecan-based therapy.

Results: Drg1 expression was identified in all metastatic tissue samples. There was a trend for unilobar metastases with high Drg1 expression ($P = 0.07$) and a suggestion of improved 2-year survival (82.4% versus 69.6%, $P = 0.148$). High Drg1 expression suggested irinotecan resistance ($P = 0.07$).

Conclusions: In colorectal cancer, Drg1 expression may be associated with a less aggressive, indolent colorectal cancer. High Drg1 may also be associated with relative resistance to irinotecan. The role of Drg1 in malignancy continues to be defined.

Colorectal carcinoma is the second most deadly cancer in the United States, with an estimated more than 146,000 new cases expected to be diagnosed in 2004 (1). Over the past several years, therapeutic options for patients with colorectal cancer have increased substantially (2). However, despite these improvements, metastatic colorectal cancer remains incurable. Efforts to better understand the biological basis for disease progression may explain the wide variability observed in patients and thus may provide important clinically relevant insights into disease management.

Differentiation-related gene 1 (*Drg1*), also known as N-*myc* down-regulated gene 1 (*NDRG1*), is a recently identified gene that is strongly induced during the differentiation of colon carcinoma cell lines (3). Drg1 mRNA was identified in all tissues examined with the highest expression in the brain, prostate, kidney, and intestine (4). The function of *Drg1* remains unknown. This gene appears to be the first of four members of a highly conserved gene family, encoding for proteins that are conserved between mouse and human but without homology to other known proteins (4–6). The protein encoded by the *Drg1* gene has a molecular weight of 43 kDa, possesses three unique 10-amino-acid tandem repeats at the COOH-terminal end, has multiple phosphorylation sites, and has been shown to be phosphorylated by protein kinase A (7). Its expression appears to be regulated by multiple signals, including hypoxia (8), DNA damage (9), and various differentiating agents and chemicals (10–12).

Drg1 expression appears to be down-regulated in malignancy. In prostate cancer, low Drg1 expression correlates with higher grade of prostate cancer, development of metastases, and with poor patient survival (13). In breast cancer, low Drg1 expression is associated with more advanced cancer stage and worse survival (14). In colon cancer, Drg1 mRNA levels appear to decrease with progression from normal colonic epithelium to carcinoma (3). Drg1 protein is observed in the cytoplasm and enters the nucleus upon DNA damage. It has been identified as a putative suppressor of metastases, virtually absent in colon cancer cells lines derived from metastatic lesions and present at higher levels in colon cancer cell lines derived from primary tumors (4). The overexpression of Drg1 in colon cancer cell lines correlated with colon cancer cell differentiation, an increase expression of E-cadherin and carcinoembryonic antigen, and a reduction in the development

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Received 11/29/04; revised 2/4/05; accepted 2/9/05.

Grant support: National Cancer Institute, Bethesda, MD grant R01 CA 067819 (G.K. Schwartz), American Society of Clinical Oncology young investigator award 2001 (M.A. Shah), and CALGB investigator award 2001 (M.A. Shah).

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of metastases, both in an *in vitro* Matrigel assay and in an *in vivo* colon cancer xenograft model.

To date, the cumulative data based on preclinical *in vitro* and *in vivo* studies suggest that *Drg1* may be an important gene that is suppressed in the development of colon cancer and in its metastatic spread. We performed this study to better evaluate the clinical relevance of Drg1 in a uniform population of patients with colon cancer. In addition, we have shown that Drg1 overexpression results in irinotecan resistance in colon cancer cell lines *in vitro* and *in vivo* and that Drg1 is transcriptionally induced by irinotecan (15). For us to determine *de novo* Drg1 expression and its clinical relevance, it was important to identify a population of colorectal cancer patients that was irinotecan naive. We therefore examined Drg1 expression in a prospectively collected tissue bank of colorectal cancer liver metastases from patients enrolled in a trial of systemic fluorouracil/leucovorin with or without intrahepatic floxuridine for patients followed by hepatic resection from 1991 to 1995 (16). Because all patients were enrolled before the approval of irinotecan in the United States, they were not exposed to irinotecan during the course of the study and at the time of tissue procurement.

Overexpression of Drg1 in HCT-116 human colon cancer cells is associated with resistance to irinotecan therapy. The growth characteristics were notably similar to the parental cells in no-drug conditions, suggesting that overexpression of Drg1 was not associated with intrinsic differences in proliferation. Conversely, underexpression of Drg1 is associated with increased irinotecan sensitivity (15). We therefore did a second analysis on our study population and evaluated whether basal Drg1 expression would be predictive of response to subsequent irinotecan-based chemotherapy at the time of first recurrence following liver resection.

Altogether, these evaluations represent the greatest clinical experience with Drg1 available to date, and we believe they provide insight into the potential biological function of Drg1 with regard to both the natural history of colon cancer progression and its potential role in irinotecan resistance.

Materials and Methods

Immunohistochemistry. Drg1 rabbit polyclonal antibody (courtesy of Theresa Commes, UPR Centre National de la Recherche Scientifique, Universite Montpellier II, France) was used at 1:8,000 final dilution. Briefly, sections were immersed in 0.01 mol/L citric acid (pH 6) and boiled in a microwave oven for 15 minutes. After cooling to room temperature, sections were incubated with 10% normal goat blocking serum for 30 minutes followed by the primary antibody and overnight incubation at 4°C. After washing, biotinylated goat anti rabbit IgG (Vector Laboratories, Burlingame CA) was applied at 1:1,000 dilution for 30 minutes followed by avidin-biotin complex (Vector Laboratories) at 1:25 dilution. Diaminobenzidine was used as the final chromogen and hematoxylin as the nuclear counterstain. Both positive and negative controls were run at the same time of each experiment. Cytoplasmic/plasma membrane staining was considered a specific staining and results were recorded as percent of tumor cells reactive with the antibody, as estimated from examining different fields throughout the entire tissue section. The plasma membrane/cytoplasm staining was notably and remarkably uniform in positive cells, both within an individual sample and among the cohort of patients. Therefore, to describe differences in Drg1 protein expression from case to case, it was not necessary to differentiate Drg1 staining intensity but rather to quantitate the percentage of positive-staining cells.

Data were recorded in a continuum as the percentage of tumor cells stained. The staining was reviewed by two pathologists (M.D. and C.C.) for a consensus assessment for each antigen. The analysis was done without knowledge of the treatment arm of study the patient was randomized to or of patient survival.

Patients. The patient population included a population of 131 patients that were enrolled from 1991 to 1995 in a random assignment study of systemic chemotherapy with fluorouracil and leucovorin versus intrahepatic chemotherapy with floxuridine in addition to systemic chemotherapy with fluorouracil and leucovorin. In the clinical trial, all patients had metastatic colorectal cancer to the liver without other sites of metastatic disease and a total of 156 patients were randomized (16). Following complete resection, 74 patients were randomly assigned to receive intrahepatic pump chemotherapy with floxuridine and dexamethasone in addition to systemic chemotherapy with fluorouracil and leucovorin. Eighty-two patients were assigned to receive fluorouracil and leucovorin alone. Tissue from 25 patients were not available; hence, the analyses were based on the remaining 131 patients. For the exploratory analysis of Drg1 expression and irinotecan sensitivity, patients from this cohort who developed a recurrence and received irinotecan-based therapy were identified. As this was a retrospective and hypothesis generating analysis, we used time on irinotecan-based therapy as a surrogate for sensitivity to irinotecan.

Statistics. Differences in Drg1 expression (low versus high) and clinical and laboratory variables were compared using the χ^2 test. Overall survival time was calculated from the time from surgery to last follow-up or date of death. Progression-free survival was calculated for patients who recurred as the time from surgery to time of first recurrence and time from surgery to last follow-up/death for patients who did not recur. Univariate survival analysis (overall and progression free) was estimated using Kaplan-Meier methods. P-values were calculated using the Cox proportional hazards model. We compared mean times between groups (i.e., time on irinotecan treatment for Drg1 high versus low) using the *t* test.

To dichotomize Drg1 expression, we use a maximal χ^2 approach (17). A Drg1 cut point value of 30 is associated with the (uncorrected) minimum $P = 0.24$. Although this value is not significant, it is the point at which we see the greatest difference in survival.

Results

Drg1 in colorectal cancers. We characterized the expression of Drg1 in a prospectively identified cohort of 131 patients with colon cancer metastatic to the liver. Table 1 describes patient characteristics of the study population. Drg1 staining in the liver lesions varied from 5% to 80%, with a mean of 40.2% and a median of 40% (see Fig. 1). Immunohistochemical staining was primarily cytoplasmic and cell membrane and showed nuclear exclusion (see Fig. 2). There appeared to be an increased staining on the periphery of tumor lesions.

Based on the cut point analysis, Drg1 expression of 30% best divided the group of patients in to high versus low Drg1 staining, such that high Drg1 is defined as >30% cells staining and low Drg1 is defined ≤30% cells staining for the Drg1 antigen. Table 2 describes the relation of Drg1 staining to numerous categorical tumor variables and patient characteristics. There was no correlation between Drg1 staining and sex, number of metastatic lesions, type of surgery performed, nor site or stage of the primary tumor. There was no correlation between Drg1 expression and the expression of other molecular markers including p53, epidermal growth factor receptor, and thymidilate synthase. There was a trend for Drg1 expression to be higher in patients with unilobar metastases ($P = 0.07$).

Table 1. Characteristics of the study population

Categorical variables	*n* = 131 (%)
Sex	
Female	52 (39.7)
Male	79 (60.3)
Age	
Mean ± SD	57.9 ± 10.6
Primary tumor	
Duke's B	44 (33.6)
Duke's C	87 (66.4)
Tumor distribution	
Unilobar	95 (72.5)
Bilobar	36 (27.5)
Number of liver metastases	
1	50 (38.2)
2-4	55 (42.0)
>4	26 (19.8)

Table 3 demonstrates the low correlation between Drg1 expression and several continuous laboratory and tumor parameters, including tumor size, time to recurrence, lactate dehydrogenase, and preoperative and postoperative carcinoembryonic antigen. Also of note, the primary tumor was available in a subset of 26 patients, all of whom had their primary tumor resected concurrently with the resection of the hepatic metastases. In this subset, Drg1 expression in the primary tumors varied from 20% to 70% and was not significantly different nor convincingly similar to the expression of Drg1 in the liver metastasis (data not shown). In the primary tumor, the adjacent normal colonic epithelium consistently had 90% to 100% Drg1 expression, similarly cytoplasmic and cell membrane. Comparatively, Drg1 staining in the primary tumor was always less than the adjacent normal colon and was significantly more heterogeneous and patchy.

Although Drg1 expression does not seem to significantly correlate with patient survival or time to progression for the entire population or in either treatment arm individually, there are several consistent trends suggesting high expression of Drg1 is associated with indolent tumor and with improved survival. As shown in Fig. 3., the Kaplan-Meier survival curve for high Drg1 expression is consistently superior than that of low Drg1 throughout the follow-up period (median follow-up for survivors 114 months; range, 54.7-147.3 months). The 2-year survival for high Drg1 expression was 82.4% [95% confidence interval (95% CI), 73.8-91.1%], whereas the 2-year survival for low Drg1 was 69.6% (95% CI, 57.6-81.7%; $P = 0.148$). The 5-year survival for high Drg1 expression was 52.0% (95% CI, 40.7-63.3%), whereas the 5-year survival for low Drg1 expression is 48.2% (95% CI, 35.17-61.3%). Similarly, when examining the cohort of patients with negative resection margins treated with systemic therapy alone, the median survival for patients with high Drg1 staining was 91.4 months (95% CI, 42.3-122.0 months) whereas, for patients with low Drg1 staining, median survival was 47.8 months (95% CI: 14.8-84.9 months; $P = 0.11$). This substantial difference was much less apparent for the group of patients that were randomly assigned to systemic therapy plus intrahepatic floxuridine: high Drg1 patients had median survival 66.7 months (95% CI, 36.6 to not estimable) versus 70.0 months (95% CI, 47.8 to not estimable) for low Drg1 patients ($P = 0.97$).

In multivariate analysis without the inclusion of Drg1 expression analysis, we previously reported in this patient population that thymidilate synthase was the most important predictor of overall survival and that there was a treatment arm-thymidilate synthase interaction, such that high thymidilate synthase expression was associated with a particularly low overall survival in the systemic treatment–only arm of the randomized study (18). The inclusion of Drg1 in this analysis did not change this multivariate analysis.

Drg1 and sensitivity to chemotherapy. Fifty-eight patients had received previous fluorouracil-based chemotherapy. Figure 4 shows the distribution of Drg1 expression in patients who have and have not received previous fluorouracil treatment. The distribution of Drg1 expression is not significantly different suggesting that fluorouracil-based chemotherapy does not significantly change Drg1 expression.

Of the total 131 patients examined, 83 patients developed a recurrence, of whom 37 patients received irinotecan-based therapy (data available in 35 patients). In this subset, patients with high Drg1 expression remained on irinotecan-based therapy for a mean of 6.8 months (range, 1.0-13.6 months), whereas those patients with low Drg1 expression remained on irinotecan-based therapy for a mean of 9.3 months (range, 2.8-27.1 months). By treatment arm, patients who were randomly assigned to receive systemic adjuvant chemotherapy alone remained on irinotecan-based therapy for 7.4 and 7.5 months, respectively, for Drg1 low and high groups. However, the

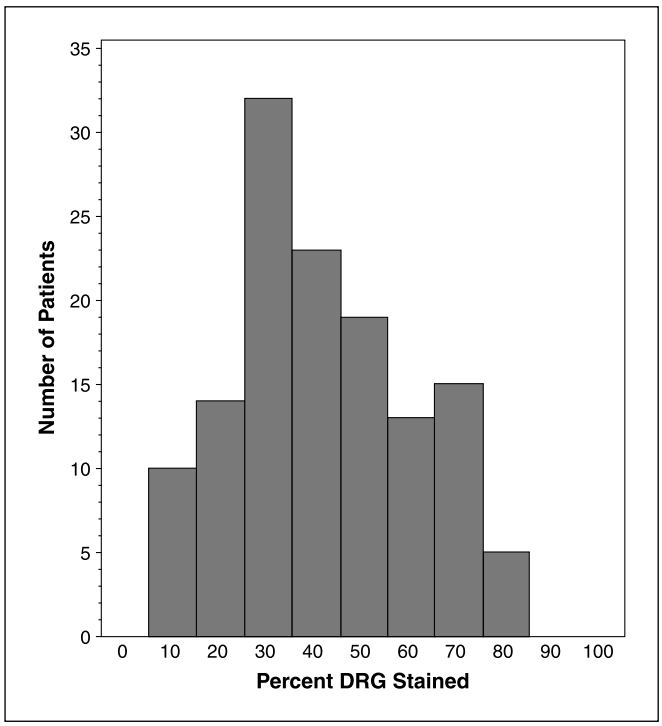

Fig. 1. Histogram of Drg1 expression. Liver metastases of 131 patients with metastatic colon cancer to the liver. Drg1 staining in the liver lesions varied from 5% to 80%, with a mean of 40.2% and a median of 40%.

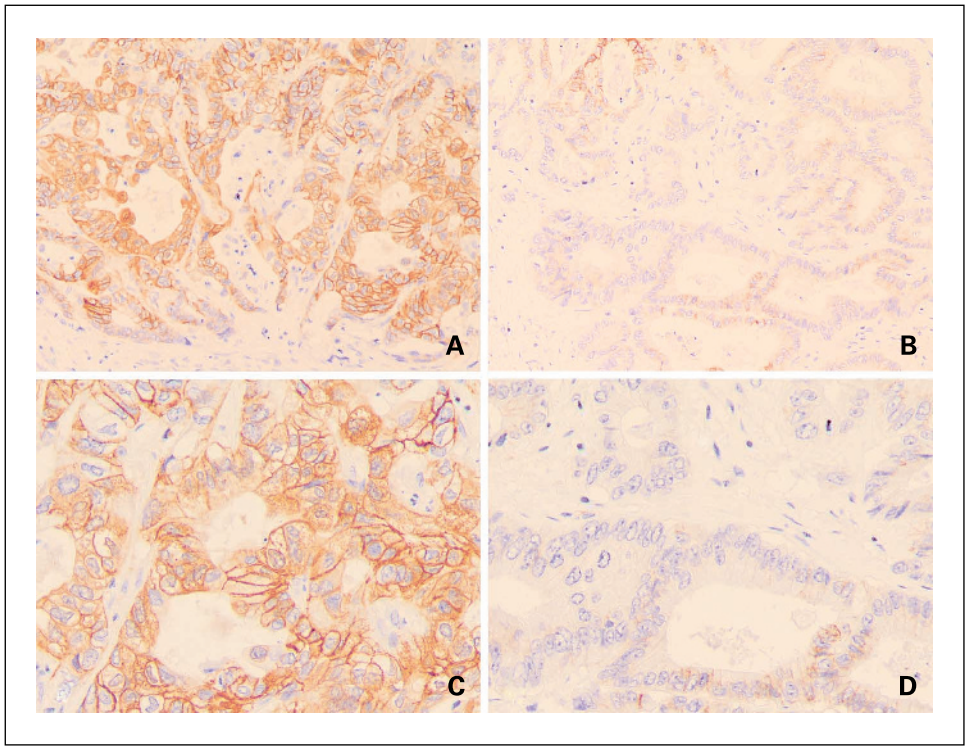

Fig. 2. Low- and high-power views of Drg1 staining in liver metastases. *A* and *C,* representative tissue biopsy with high Drg1 staining; *B* and *D,* representative tissue biopsy with low Drg1 expression. *A* and *B,* 200× magnification; *C* and *D,* 400× magnification.

patients randomly assigned to receive intrahepatic pump floxuridine along with systemic fluorouracil and leucovorin remained on irinotecan-based chemotherapy following recurrence for 13.0 and 6.2 months, respectively, for Drg1 low and high groups ($P = 0.07$). We found no association between these patients and baseline clinical or tumor characteristics.

Discussion

Drg1 is a recently identified protein that is highly conserved and may be important in the pathogenesis of metastases formation and cancer progression. It was identified in colon cancer cell lines comparing the difference in gene expression between cell lines derived from metastatic lesions and from primary tumors. It is transcriptionally induced by irinotecan and its suppression may be important in increasing irinotecan sensitivity (15). This study represents the largest clinical characterization of Drg1 in malignancy to date, in a population of patients accrued to a clinical trial from 1991 to 1995. These patients represent a very unique cohort of patients in which to examine the potential role of *de novo* Drg1 expression in the natural course and history of colorectal cancer, in that a similar population of patients with metastatic disease, specifically one that has not been exposed to irinotecan, would be virtually impossible to identify in the modern treatment era.

Preclinical data suggest that Drg1 may suppress colon cancer metastases by inducing their differentiation and reversing the metastatic phenotype. Indeed, constitutive overexpression of Drg1 in colon cancer cell lines that had low basal expression was associated with a significant decrease in migration in Matrigel and a reduction in liver metastases following splenic injection in a mouse colon cancer model compared with parental and vector control cell lines (4). These investigators identified *Drg1* as a gene that was virtually absent in cell lines derived from colon cancer metastatic sites and present at high levels from colon cancer cell lines derived from primary tumors. When examining Drg1 expression by Northern blot analysis in five metastatic clinical samples, they observed that the Drg1 expression was undetectable in three and substantially down-regulated in two metastatic lesions (4). However, in contradiction to these preclinical data, our data from clinical specimens clearly indicate that Drg1 protein is indeed expressed in metastatic colon cancer lesions by immunohistochemistry. The expression does vary from 5% expression to 80% expression; however, all 131 patients examined had expression of Drg1 in their metastatic liver lesions. Recalling that the conclusions were made primarily from the difference in expression of Drg1 between cell lines derived from primary and metastatic lesions, these clinical data also highlight the lack of correlation between relatively artificial *in vitro* cell culture systems and *in vivo* systems and further suggest the importance of clinical correlation and validation to preclinical hypothesis generating data. This discrepancy may also, in part, be explained by the fact that our study population was metastatic to begin with, thus perhaps highlighting a subtle difference of the role of Drg1 in early-stage cancers versus metastatic disease.

Bandyopadhyay et al. suggest that Drg1 expression is associated with earlier stage of disease in a cohort of prostate and breast cancer patients (13, 14). In our study, the subset of patients for whom the primary tumor was available also had synchronous liver metastases (i.e., patients were stage IV at the time of resection). We noted that Drg1 expression was not significantly different between the primary and metastatic lesion. This is consistent with a previous report also demonstrating no appreciable difference in Drg1 expression by Northern blot analysis in 36 clinical specimens of primary

Table 2. Drg1 staining characteristics and their relation to tumor characteristics

Variables	Low Drg1, *n* (%)	High Drg1, *n* (%)	*P* (χ^2 test)
Sex			
Female	21 (38)	31 (41)	0.66
Male	35 (62)	44 (59)	
Primary tumor			
Duke's B	15 (27)	29 (39)	0.15
Duke's C	41 (73)	46 (61)	
Primary site			
Colon	46 (82)	55 (73)	0.24
Rectum	10 (18)	20 (27)	
Tumor distribution			
Unilobar	36 (64)	59 (79)	0.07
Bilobar	20 (36)	16 (21)	
Resection margin			
(−)	48 (86)	63 (84)	0.79
(+)	8 (14)	12 (16)	
Prior chemotherapy			
No	26 (46)	33 (44)	0.78
Yes	30 (54)	42 (56)	
Type of surgery			
Lobectomy	23 (41)	31 (41)	0.88
Segmentectomy	13 (23)	14 (19)	
Trisegmentectomy	17 (30)	24 (32)	
Wedge Resection	3 (4)	6 (8)	
No. metastases			
1	23 (41)	27 (36)	0.82
2-4	22 (39)	33 (44)	
>4	11 (20)	15 (20)	
Thymidilate synthase			
(−)	45 (80)	67 (89)	0.15
(+)	11 (20)	8 (11)	
p53			
(+)	28 (50)	43 (57)	0.40
(−)	28 (50)	32 (43)	
Epidermal growth factor receptor			
(−)	47 (84)	62 (86)	0.73
(+)	9 (16)	10 (14)	

Table 3. Correlation of Drg1 with laboratory variables

Variables	Low Drg1, mean	High Drg1, mean	*P* (*t* test)
Age (y)	58	58	0.74
Disease-free interval	11	12	0.71
Largest tumor size	4.5	4.4	0.79
Carcinoembryonic antigen preoperative	70.4	125.4	0.57
Carcinoembryonic antigen postoperative	4.9	9.0	0.29
Lactate dehydrogenase	332.4	301	0.52
Serum alkaline phosphatase	94.8	96.7	0.79
Aspartate aminotransferase	91.0	74.3	0.56
Serum albumin	4.1	4.1	0.81
White cell count	10.0	9.1	0.33

and metastatic breast cancer lesions (4). There was no correlation with Drg1 expression in the metastatic lesion and the stage of the primary tumor. Also consistent with previous reports, Drg1 expression in the primary tumor was always less than adjacent normal colon, although the staining pattern and level of expression in the tumor varied widely. These data suggest that although perhaps associated with less metastatic potential, even primary tumors that have high Drg1 expression have the ability to metastasize.

Although we did not observe a relation between Drg1 and the number of metastatic lesions in the liver, there was a suggestion that high Drg1 expression is associated with unilobar liver metastases. There was also a suggestion that high Drg1 expression may be associated with an improvement in colon cancer survival at 2 years, but this was not sustained at 5 years. In patients with metastatic liver resections with negative margins, treated with systemic fluorouracil alone, high Drg1 expression was associated with a median survival of 90 months (versus 48 months for low Drg1 expression). Altogether, these data suggest that patients who had a high Drg1 expression had tumors that were more indolent and slow growing. This phenotypic finding is indeed consistent with the preclinical data that suggests that Drg1 expression is associated with a less aggressive phenotype (as shown by reduced migration through Matrigel and fewer liver metastases in the mouse model; ref. 4).

Despite its widespread use in colorectal cancer and numerous other malignancies, the mechanisms of resistance to irinotecan remain unclear. Topoisomerase I is the target of irinotecan, and point mutations in this enzyme have been identified in resistant prostate and lung cancer cell lines (19, 20). However, topoisomerase I levels have not correlated with irinotecan

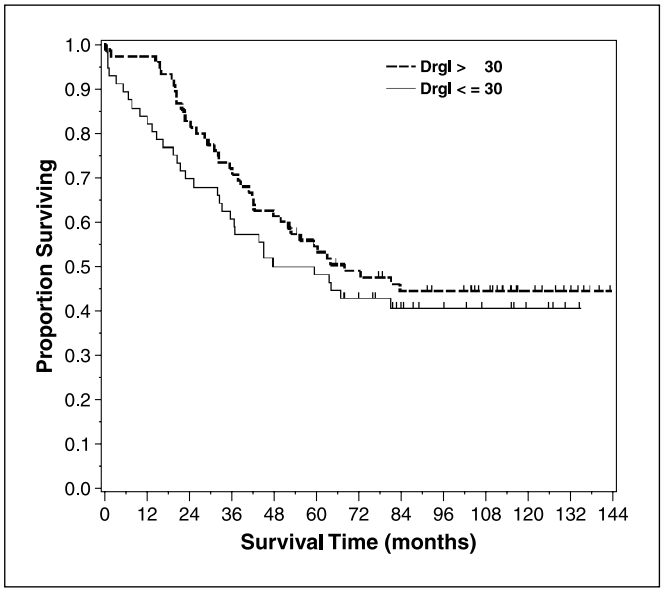

Fig. 3. Drg1 expression and association with overall survival ($P = 0.28$, Cox regression model). To dichotomize Drg1 expression, we use a maximal χ^2 approach (17). Although the Drg1 cut point value of 30% is not significant, it is the point at which we see the greatest difference in survival.

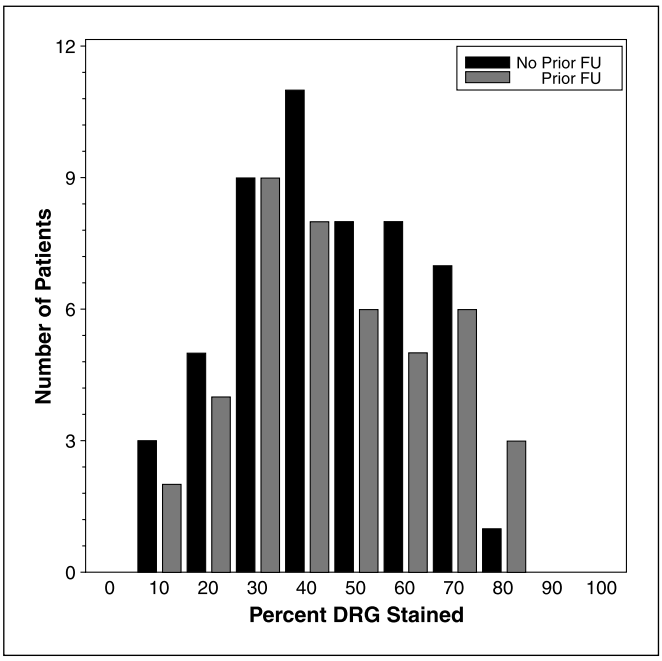

Fig. 4. Drg1 distribution in patients that did and did not receive fluorouracil-based therapy before resection of the metastatic liver lesion.

resistance (21). Having an intact G_1 and G_2 cell cycle checkpoint (22), loss of p53 (23), the presence of the DNA mismatch repair gene *hMLH1* (24), and the overexpression of the ABCG2 transporter (25) all have been reported mechanisms to influence irinotecan sensitivity.

We previously reported that Drg1 expression may be in part responsible for resistance to irinotecan-based therapy (15). We explored this hypothesis first in a subset of patients that developed recurrence and proceeded to receive irinotecan-based systemic chemotherapy. In this analysis, patients with low Drg1 expression consistently remained on irinotecan-based therapy seemingly longer than patients with high Drg1 expression, suggesting increased sensitivity to irinotecan. This difference was most dramatic in the patients that were randomized to receive systemic fluorouracil and leucovorin with intrahepatic floxuridine therapy. Although this represents an ad hoc retrospective analysis, these data are consistent with the preclinical data and support the hypothesis that Drg1 expression may be associated with irinotecan resistance. What is not tested in this data set is whether subsequent irinotecan therapy induced Drg1 expression and, if so, at what point this occurred relative to irinotecan therapy and irinotecan failure. This will require future prospective clinical evaluation and analysis. Furthermore, we previously reported that irinotecan resistance can be overcome by the sequential and timely administration of the cell cycle modulator, flavopiridol (26), and that this potentiation may be related to Drg1 modulation (15). In view of this, we have begun to examine pretreatment and post-treatment tumor biopsies in patients receiving this combination (27).

This study represents a large and systematic evaluation of Drg1 in a cohort of patients with metastatic colon cancer to the liver before the era of irinotecan-based therapy. In colon cancer, in contradiction to previous preclinical reports, loss of Drg1 expression does not appear to be solely responsible for the development of metastases. However, its expression may indeed suggest a more indolent course of disease, in support of the previous studies. In this population of patients, the number of metastases resected, and other cellular markers including thymidilate synthase, seemed more predictive of patient survival. This may not be surprising as these patients were treated in the adjuvant setting from 1991 to 1995 where the only cytotoxic drug available to patients was fluorouracil. Our study also supports the preclinical data that suggests high basal Drg1 expression is associated with relative irinotecan resistance. Examining a separate cohort of patients in the current era may give us better insight as to the role of Drg1 with regard to chemotherapy resistance to irinotecan. The modulation of Drg1 and its consequence with regard to irinotecan resistance is continuing to be explored.

Based on this study and the published literature to date, it seems that Drg1 may have significant function relative to the malignant phenotype. High expression of Drg1 appears to be associated with differentiation, earlier stage of disease, and with an indolent course of metastatic disease. However, high expression of Drg1 also appears to be associated with relative resistance to irinotecan chemotherapy. Further carefully planned clinical studies are required to clarify these potential roles of this new protein. The role of Drg1 in malignancy continues to be defined.

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