Single-Cell Transcription Site Activation Predicts Chemotherapy Response in Human Colorectal Tumors

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Abstract

Candidate gene and pathway approaches, and unbiased gene expression profiling, have identified marker signatures predictive of tumor phenotypes, such as drug sensitivity and invasive or metastatic potential. However, application of such information to evaluation of tumors in the clinic is limited by cell heterogeneity in the tumor. We have developed a novel method of fluorescence *in situ* hybridization (FISH) that can detect transcriptional activation of individual genes at their site in single cells in the interphase nucleus. A major obstacle in the treatment of colorectal cancer is relative insensitivity to the chemotherapeutic agent 5-Fluorouracil (5-FU). Here, we have developed a sensitive approach to predict relative sensitivity of colorectal cancer cells to 5-FU, using FISH with probes targeted to nascent mRNAs to measure the number of individual cells with active transcription sites for a panel of candidate genes. These results reveal that the transcriptional status of four key genes, *thymidylate synthase* (*TYMS*), *MORF-related gene X* (*MRGX*), *Bcl2-antagonist/killer* (*BAK*), and *ATPase, Cu^{2+} transporting β polypeptide* (*ATP7B*), can accurately predict response to 5-FU. As proof of principle, we show that this transcriptional profile is predictive of response to 5-FU in a small number of patient colon tumor tissues. This approach provides a novel ability to identify and characterize unique minor cell populations in the tumor that may exhibit relative resistance to chemotherapy. [Cancer Res 2008;68(13):4977–82]

Introduction

5-Fluorouracil (5-FU) is the most commonly used agent in combination therapy for colorectal cancer in either an adjuvant or advanced stage setting (1). Although stage is a significant predictor of likely outcome, cellular and molecular markers of sensitivity to 5-FU, or disease-free or overall survival, have been identified for each stage. Among these are levels of thymidylate synthase and thymidine phosphorylase, two enzymes intimately related to 5-FU metabolism (2–4). The presence of microsatellite instability has also been linked to 5-FU response (5, 6). Finally, the presence of a wild-type p53 gene (7–9), especially when coupled with amplification and/or elevated expression of the c-*myc* gene (10, 11), correlates with a favorable response to 5-FU.

More recently, unbiased approaches that use gene expression profiling have characterized response to drugs and prognosis. With regard to colorectal cancer, heterogeneous responses to 5-FU (12), camptothecin (12), and oxaliplatin (13) were identified in a panel of 30 cell lines, and microarray analysis was used to identify gene expression profiles predictive of relative sensitivity to these drugs. The predictive value of this approach was better than other molecular markers that have been reported (12).

Regardless of the method used to identify clinically useful markers of drug response, all approaches must eventually deal with the fact that tumors are highly heterogeneous. Only a minor proportion of the cells may be relatively drug resistant or have other important clinical phenotypes, such as propensity to invade or metastasize. Because these cells cannot be identified histologically, alternate means are necessary for their detection. This is not only of major clinical importance, but the distribution of such cells in relation to important features of the tumor, such as the invasive front or the proximity to blood supply, provide significant insight into the cell biology of tumor formation and progression. Although immunohistochemistry can provide such information, it is limited by the availability of appropriate antibodies, as well as in the number of distinct gene products that can be identified simultaneously. An alternate approach is to examine gene expression patterns of individual cells using fluorescence *in situ* hybridization (FISH). This could also prove to be crucial in assaying biopsy samples that contain very small deposits of cancer cells, below the detection threshold of assays such as microarrays and Northern blots, which measure total RNA for a population of cells (14). Earlier work in our laboratory has shown that FISH can detect individual nascent mRNAs in the nucleus as well as single mRNA molecules in the cytoplasm with high spatial resolution (15).

We have reported a method of FISH that can identify whether a particular gene is transcriptionally active in cultured cells (16), or in formalin-fixed tissue (17). Moreover, using multiple probes of distinct fluorescence emissions, and combinatorial multiplexing of such probes, we have shown that activation of 10 genes in single cells can be assayed simultaneously (16). In this report, we used this methodology in both tissue culture and fixed tissue to define a subset of genes that differentiates between colorectal tumor cells that are relatively sensitive or resistant to 5-FU.

Materials and Methods

Cell culture. DLD, HCT15, SW837, SW620, HCT116, RW2982, and SW403 cell lines with documented responses to 5-FU were provided by JM Mariadason (Montefiore Medical Center, Bronx, NY), grown in MEM (Cellgro), supplemented with 10% fetal bovine serum (Invitrogen), 1% penicillin/streptomycin (Invitrogen), 100 μmol/L nonessential amino acids (Sigma), and 10 mmol/L HEPES buffer (Invitrogen) in a humidified incubator at 37°C with 5% CO_2.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Oligonucleotide probe design and synthesis. Probes for FISH were designed using OLIGO-6.0 software (Molecular Biology Insights), and specificity was verified through the National Cancer Institute GeneBank nucleotide-nucleotide BLAST program. For each target nascent transcript, 4 50-mer DNA probes were synthesized containing 4 to 5 modified thymidine bases (Supplementary Fig. S1) conjugated to either Cy3 or Cy5 succinimidyl ester fluorescent dyes (GE Healthcare).

Patient tissue samples. Tissue microarrays (TMA) containing core biopsies of paraffin-embedded tissues from 15 anonymous colon cancer patients in triplicate were purchased (US Biomax). Paraffin-embedded tissue samples with known outcomes were obtained from seven patients who had undergone treatment for colon cancer at the Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA (approved by the Thomas Jefferson University Institutional Review Board).

RNA FISH in cultured cells and paraffin-embedded tissues. Cells were grown on glass coverslips, extracted with Triton X-100, fixed with 4% paraformaldehyde, and hybridized with 20 ng of labeled probe as described (16). Paraffin-embedded tissue FISH was performed as described (17).

Detection of transcription sites. Fluorescent signals were detected with an epifluorescence Olympus AX70 microscope, UApo 40X, 1.35NA and PlanApo 60X, 1.4NA objectives, and a CoolSNAP-HQ CCD camera (Photometrics) using filters for 4′,6-diamidino-2-phenylindole (DAPI), FITC, Cy3, and Cy5 (Chroma Technology). Stacks of images were acquired with a

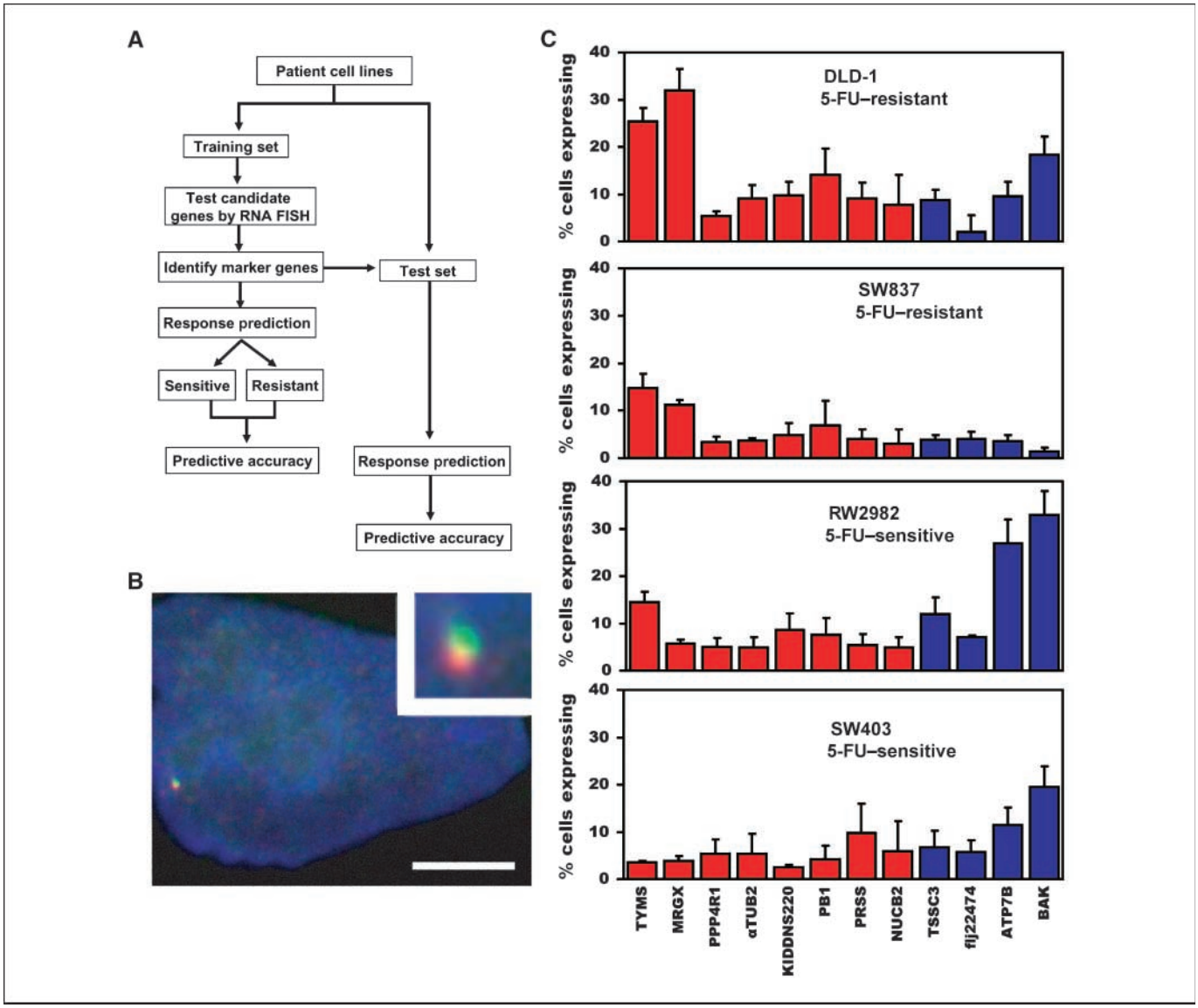

Figure 1. Defining markers of 5-FU response in human colorectal tumor cell lines using single-cell profiling of transcription site activation. *A,* flowchart of the strategy used to define a predictive model for response to 5-FU–based chemotherapy. Candidate genes were selected from gene expression profiles of each human colorectal adenocarcinoma cell line. The training set of cell lines selected represents the extremes of sensitivity or resistance to 5-FU. A transcription site activation profile of candidate genes was determined for each cell line. Using leave-one-out analysis, a predictive model that classified the training set of cell lines as resistant or sensitive to 5-FU with the highest accuracy was derived. The predictive marker genes were evaluated for their ability to accurately classify a panel of independent test cell lines as 5-FU–resistant or–sensitive in a blinded study. *B,* detection of an active transcription site for the gene *MRGX* in an individual human colorectal adenocarcinoma cell (DLD-1). Nuclei are stained with DAPI and sites of transcription are detected with fluorescent probes labeled in Cy3 (*red*) and Cy5 (*green*). *Inset,* close-up of area of nucleus with both Cy3 and Cy5 probes bound to nascent transcripts. *Scale bar,* 6 μm. *C,* transcription site activation profile of 5-FU–resistant and 5-FU–sensitive colorectal tumor cell lines as measured by FISH for nascent mRNAs. Analysis of active transcription sites for each candidate gene in individual cells provides a transcriptional profile for each cell line. *Red bars,* candidate genes correlated with 5-FU resistance; *blue bars,* candidate genes correlated with 5-FU sensitivity. *Columns,* mean for three experiments; *bars,* SE.

200-nm Z step size and analyzed using IPLab software version 3.61 (BD Biosciences). Random fields of cells were imaged to ensure that differences in numbers of active transcription sites between samples were due to differences in transcription and not due to heterogeneity in proliferation among cells within a culture or tissue sample. Transcription sites were assayed in untreated cell cultures and tissues except for samples from patients 1F, 4F, and 6F, who received 5-FU therapy before surgical resection of their tumors. Only nuclei located entirely within the imaged field were scored for presence or absence of transcription sites. Each image within a stack was analyzed separately to accurately count nuclei in close proximity. Fluorescent spots in the nucleus were identified as transcription sites based on fluorescence intensity, volume, and shape. Spots also present in the FITC channel represented autofluorescence and were not counted. Transcripts were first detected individually, using Cy3 and Cy5 probes. After identifying a four-gene signature predictive of 5-FU response, the genes were analyzed simultaneously in the same sample. Two genes correlating with resistance (*TYMS* and *MRGX*) were detected with probes labeled with one fluorophore, and two genes correlating with sensitivity (*ATP7B* and *BAK*) were detected with probes labeled with a different fluorophore. Percentage of transcription sites for each gene was calculated from the total number of transcription sites detected and the total number of nuclei detected.

Statistical analysis. Statistical tests were performed using MATLAB v7.0.1 (MathWorks). To perform logistic regression, we let P be the probability that a cell line is sensitive to 5-FU, given its gene expression profile $x = [x_1, \ldots x_n]$, where x_n is the percentage of cells containing transcription sites for gene n in cell line x. The odds of sensitivity to 5-FU are $P/(1\text{-}P)$. We parameterized the odds such that

$$ln(\frac{P}{1-P}) = w_0 + X^T w_1 \quad \text{(eq. 1)}$$

$$\therefore P = \frac{e^{w_0} + X^T w_1}{1 + e^{w_0 + X^T w_1}} \quad \text{(eq. 2)}$$

where x^T denotes the transpose of X. We used a maximum likelihood estimator that uses as an input the measured quantities x_n and outcomes for each of the training samples. The estimator then iteratively solved for P by varying the variables w_0 and w_1. The linear decision boundary could then be written as

$$ln(\frac{P}{1-P}) = w_0 + X^T w_1 = 0. \quad \text{(eq. 3)}$$

Results

To develop markers predictive of 5-FU response, we selected four colorectal adenocarcinoma cell lines representing extremes of sensitivity or resistance to 5-FU and chose a set of candidate genes including thymidylate synthase and genes that correlated highly with 5-FU response in a microarray study (12). Figure 1*A* provides an overview of the strategy. For each of the 12 candidate genes, we examined active transcription sites in individual cells using FISH (Fig. 1*B*). Our results showed differential transcription of several genes in 5-FU–sensitive or 5-FU–resistant colorectal tumor cell lines (Fig. 1*C*). We examined various combinations of these genes to identify expression signatures that correlated with either resistance or sensitivity to 5-FU.

To evaluate the predictive value of each combination of genes, we used logistic regression to build a model that predicted response of a cell line to 5-FU based on the active transcription site profile of those genes. Exhaustive combinations of the 12 potential markers for 5-FU response were used to build various models, each of which was evaluated for predictive accuracy using a training set of 4 cell lines with documented responses to 5-FU (12).

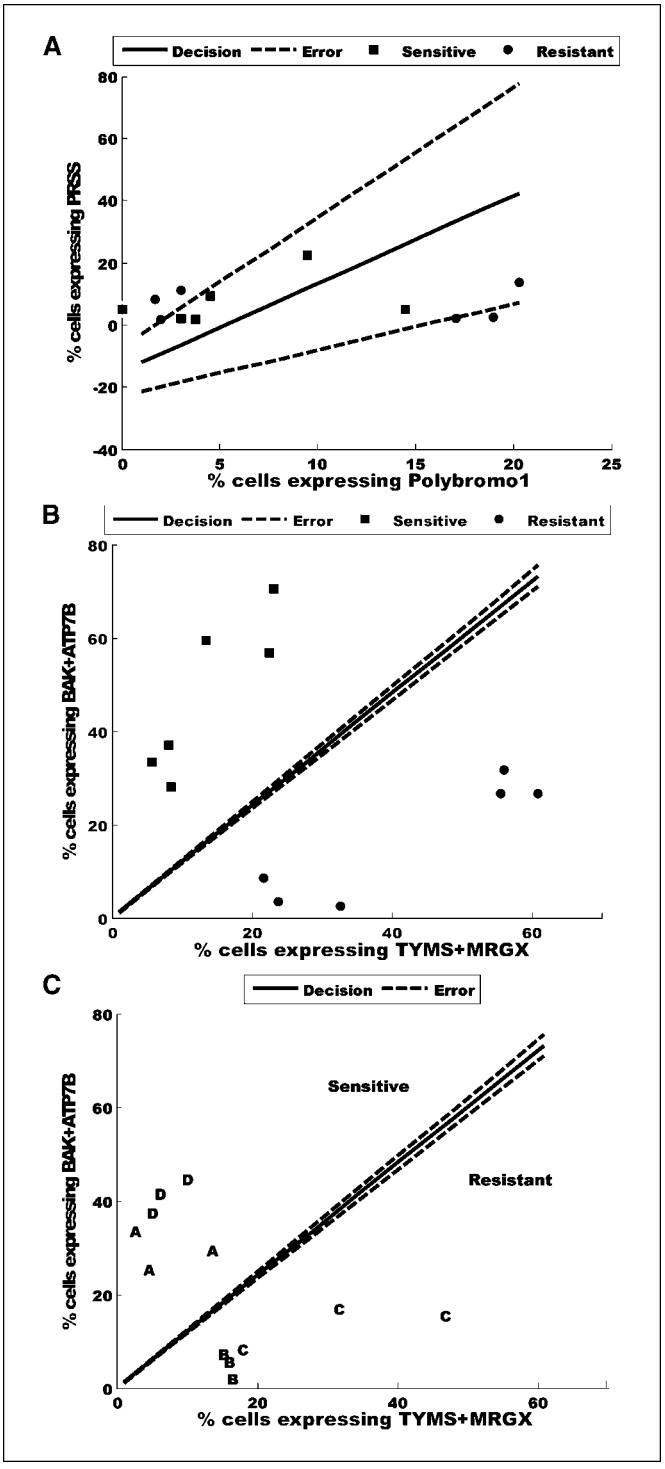

Figure 2. Chemotherapy indicator plot. *A,* two genes that are poor predictive markers of response to 5-FU treatment. *Filled squares,* cell lines known to be sensitive. *Filled cirlces,* cell lines known to be resistant. The decision line is an average of 12 decision boundaries generated from leaving out each of the 12 samples from the training set once. The large error in the decision boundary signifies the dependency of the model on a single sample in the training set. *B,* the four genes, *MRGX, TYMS, BAK*, and *ATP7B* are identified as good predictive markers of response to 5-FU treatment. *C,* performance of biomarkers in an independent set of blinded test cell lines. *Test cell lines A* and *D,* corresponding to RKO and HCT116, respectively, were classified as 5-FU–sensitive ($P = 0.05$ and $P = 0.0005$, respectively). *Test cell line B,* corresponding to SW620 was classified as 5-FU–resistant ($P = 0.023$). *C,* the fourth cell line, HCT15, was also classified as 5-FU–resistant ($P = 0.099$).

Due to the small sample size of the training set, we used leave-one-out crossvalidation to assess the accuracy of the predictive models. The transcriptional profile and the outcome of *k-1* of the *k* training samples was used to produce a linear decision boundary as outlined in the statistical methods section. The model was then used to predict the outcome of the k^{th} training sample. The process was repeated *k* times, excluding a different training sample for validation each time.

If a set of genes was not a good predictor of response to 5-FU, then the decision boundary was sensitive to each of the *k* training samples that were excluded. The result was a large variation between calculated decision boundaries, leading to poor sensitivity and specificity of the predictive model (Fig. 2*A*). Alternatively, Fig. 2*B* shows a set of genes whose expression levels yielded a model with high predictive accuracy and robustness. The variance between *k* decision boundaries calculated for each of the *k* subsets was small. A gene expression signature consisting of four genes, *TYMS, MRGX, BAK* and *ATP7B*, correctly classified the training set of cell lines as either sensitive or resistant to 5-FU (Fig. 2*B*).

This model was then used to predict the response of independent test cell lines to 5-FU. Four additional colorectal adenocarcinoma cell lines, HCT15, SW620, RKO, and HCT116, were used to test the predictive model. Analysis of these test cell lines was blinded to eliminate bias in scoring of transcription sites. Cells were scored for number of transcription sites for *MRGX, TYMS, BAK*, and *ATP7B*. Our model, consisting of these four genes, correctly predicted the response of all four test cell lines to 5-FU (Fig. 2*C*): SW620 (P = 0.023) was classified as 5-FU resistant, whereas RKO (P = 0.051) and HCT116 (P = 0.0005) were classified as 5-FU sensitive. The fourth cell line, HCT15, was classified as 5-FU resistant with somewhat lower significance (P = 0.099).

To investigate the potential of using transcription site profiling in tumors, we examined active transcription sites in tissue samples from 15 anonymous colon cancer patients on a TMA hybridized with probes for either *TYMS* and *MRGX* (Fig. 3*A*) or *BAK* and *ATP7B* (Fig. 3*B*). Although colon tumor tissues were all from patients with grade 2 colon adenocarcinomas, single-cell transcription site profiles of individual tumors revealed a large variability in the

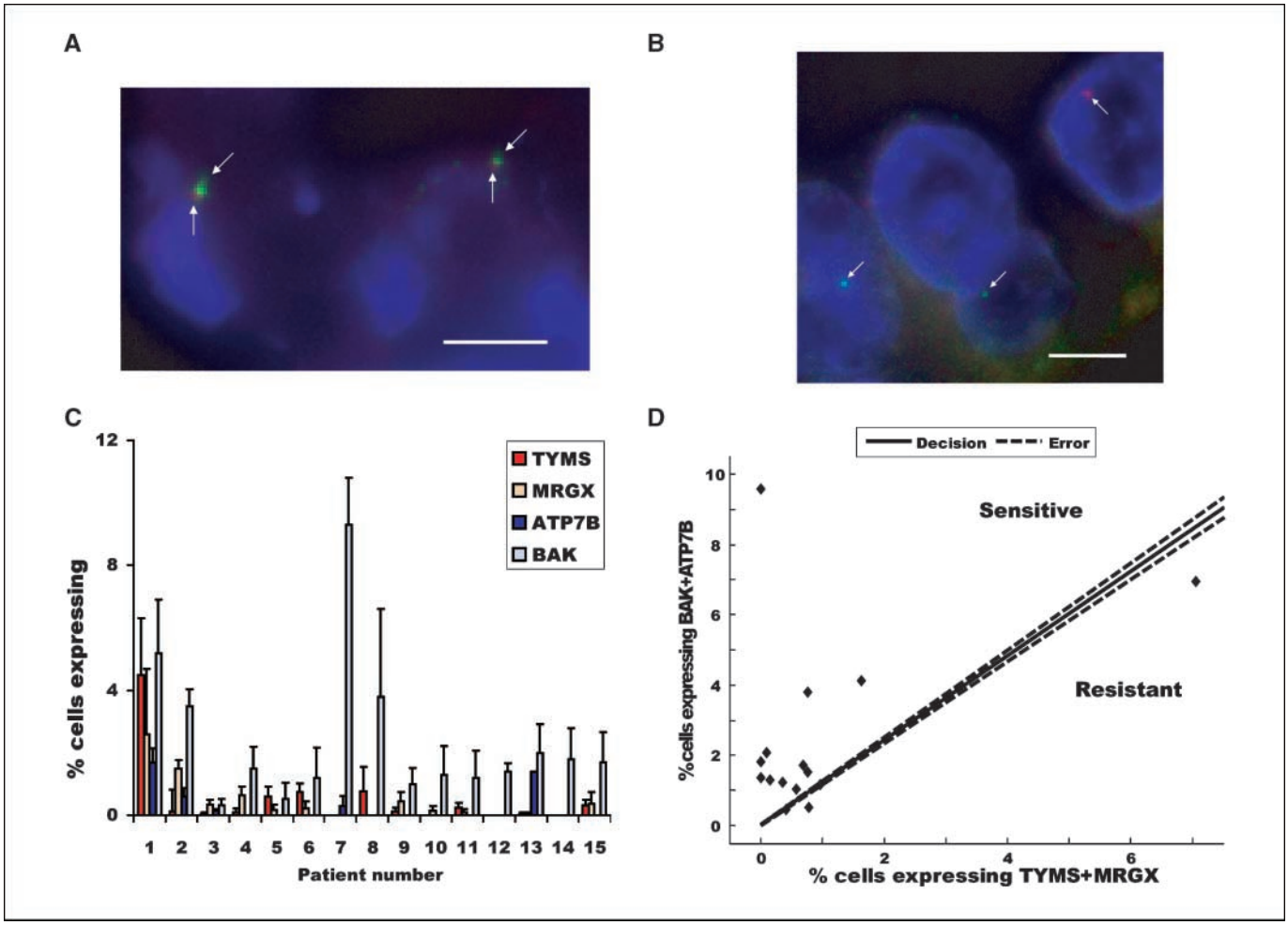

Figure 3. Detection of active transcription sites for 5-FU marker genes in paraffin-embedded human colon tumor TMA. *A,* merge of DAPI, Cy3, and Cy5 channels. Image shows DAPI-stained nuclei containing transcription sites (arrows) for *MRGX* and *TYMS. Scale bar,* 5 μm. *B,* merge of DAPI, Cy3, and Cy5 channels. Image shows DAPI-stained nuclei containing transcription sites (*arrows*) for *ATP7B* and *BAK. Scale bar,* 5 μm. *C,* active transcription site profile for 5-FU marker genes in colon tumor biopsies from individual patients as measured by RNA FISH. *Red bars,* genes correlated with 5-FU resistance. *Blue bars,* genes correlated with 5-FU sensitivity. *Columns,* mean for three sections from each individual tumor; *bars,* SE. *D,* chemotherapy indicator plot for tumors from 15 anonymous patients. *Filled diamonds,* tumor samples with unknown clinical outcomes. Our predictive model classified 11 samples as sensitive and 2 samples as resistant. The model was unable to classify the remaining two samples with significant confidence.

expression of marker genes (Fig. 3*C*). A majority of these tumor samples had high expression of the proapoptotic gene *BAK*, suggesting that these early-grade tumors would be sensitive to apoptosis induced by chemotherapeutic drugs such as 5-FU. Our predictive model classified 11 of the 15 samples as relatively sensitive (Fig. 3*D*). Two of the 15 tumors were classified as more resistant, whereas the remaining two tumors showed mixed characteristics.

To provide proof of principle that these transcription site profiles are associated with outcomes to therapy, we tested colon tumor samples from a small number of patients with known outcomes. Tissue samples were obtained from surgically resected tumors of patients undergoing treatment for colon cancer. Three patients, designated 1F, 4F, and 6F, received 5-FU–based chemotherapy before and after surgery, whereas four patients, designated 1N, 4N, 5N, and 6N, received 5-FU–based therapy only after surgery. Tissues were hybridized with probes for *TYMS, MRGX, BAK*, and *ATP7B* (Fig. 4*A*). Analysis was blinded to eliminate bias in the scoring of transcription sites. Tumors from patients 1F, 4F, and 6F had relatively higher expression of *TYMS* and *MRGX* and lower expression of *ATP7B* and *BAK*, classifying these patients as relatively less sensitive to 5-FU–based chemotherapy (Fig. 4*B*). Among these three patients, 1F had tumor recurrence after previous surgery and 5-FU–based chemotherapy, although presently has no evidence of another reccurence, whereas patients 4F and 6F both later developed metastatic disease after 5-FU–based chemotherapy. In contrast, patients 1N, 4N, 5N, and 6N had tumors with higher expression of *ATP7B* and *BAK* than *TYMS* and *MRGX*, classifying them as more sensitive to 5-FU–based chemotherapy (Fig. 4*B*). These four patients have not had a recurrence of their tumors or evidence of metastasis after surgery and 5-FU therapy, consistent with their classification as more sensitive to the drug treatment they received. On the basis of our predictive model, tumors from patients 1F, 4F, and 6F were classified as relatively resistant and tumors from patients 1N, 4N, 5N, and 6N as relatively sensitive (Fig. 4*C*).

Discussion

Assay of transcription site activation differs from gene expression profiling in two key ways. First, expression analysis by Northern blots, qRT-PCR, or microarrays measures steady-state transcript levels, whereas transcription site analysis provides data on whether the gene is on or off, essentially measuring the function of the gene as a rheostat that monitors and provides input into determining steady-state levels. As such, transcription site analysis can provide insight into the presence or absence of signals and pathways that directly activate transcription. Second, by nature of the assay, transcription site analysis provides information on individual cells, rather than the mean level of expression of a gene in a population. Thus, this method is better suited for analysis of limited amounts of tissue and for dissection of heterogeneity that likely exists in tumors. Further, this FISH-based methodology can be combined with histopathology to provide a more accurate molecular picture of the cell biology of individual tumors, such as spatial distribution and orientation of tumor cells with particular phenotypes in the context of stromal-epithelial cell interactions (17), as well as the histopathologic features of cells with particular transcription site profiles.

In this report, we have shown that transcription site profiles of key genes for which steady-state levels correlate with response to 5-FU *in vitro* (12) could be used to develop a novel approach that

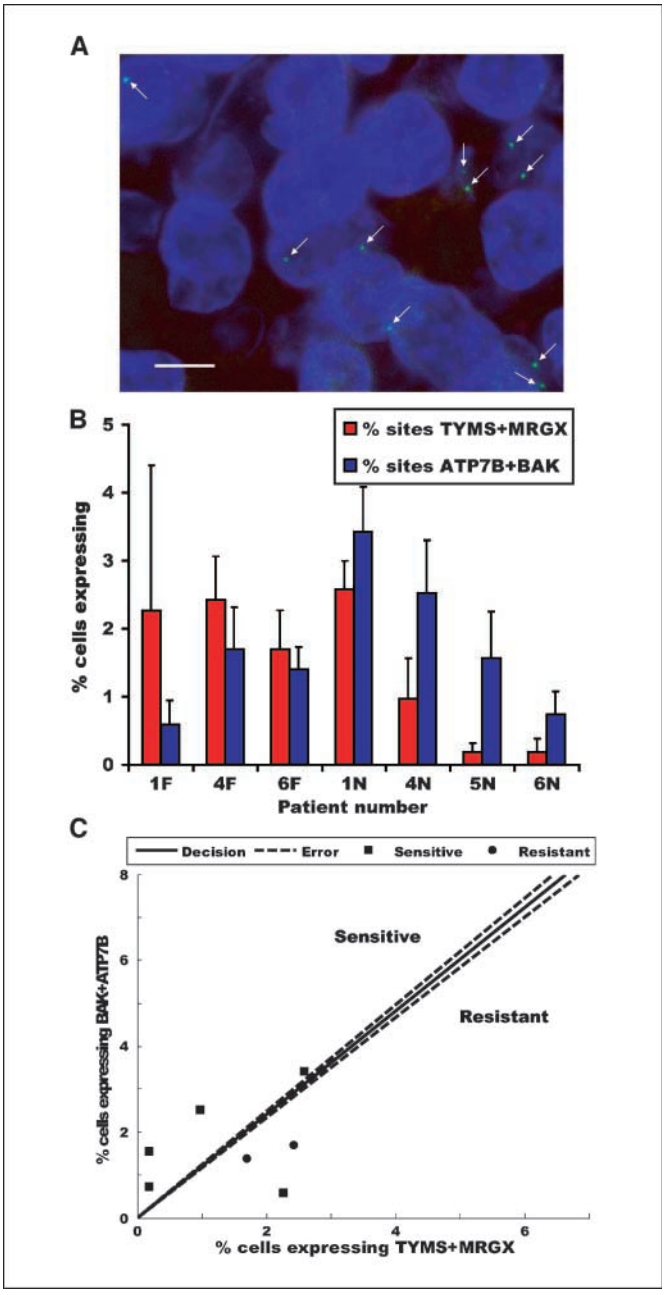

Figure 4. Prediction of response to 5-FU–based chemotherapy in colon cancer patients. *A,* active transcription sites for 5-FU marker genes in paraffin-embedded human colon tumor tissues. Image shows DAPI-stained nuclei containing transcription sites for *ATP7B* and *BAK* (both shown in green). *Scale bar,* 5 μm. *B,* active transcription site profile for 5-FU marker genes in colon tumor samples from seven patients as measured by RNA FISH. The seven patients are designated as follows: patient #1F, female, age 60 y, tumor stage $T_3N_2M_x$ (poorly differentiated adenocarcinoma); patient #4F, male, age 56 y, tumor stage $T_3N_1M_x$ (poorly differentiated mucinous adenocarcinoma); patient #6F, male, age 33 y, unknown tumor stage (metastastic adenocarcinoma); patient #1N, male, age 62 y, tumor stage $T_3N_1M_x$ (well to moderately differentiated adenocarcinoma); patient #4N, female, age 67 y, tumor stage $T_3N_2M_x$ (moderately differentiated adenocarcinoma); patient #5N, female, age 56 y, tumor stage $T_3N_2M_x$ (moderately to poorly differentiated adenocarcinoma); patient #6N, female, age 42 y, tumor stage $T_3N_1M_x$ (moderately differentiated adenocarcinoma). *Red bars,* genes correlated with 5-FU resistance. *Blue bars,* genes correlated with 5-FU sensitivity. *Columns,* mean for six fields from each individual tumor; *bars,* SE. *C,* chemotherapy indicator plot for tumors from seven anonymous patients. *Filled squares,* patients known to be sensitive. *Filled circles,* patients known to be resistant. Our predictive model classified three samples as resistant and four samples as sensitive.

predicts response of tumor cells to chemotherapy. Using cell lines representing the extremes of sensitivity and resistance, we derived a four-gene signature that independently predicted 5-FU response in test cell lines. Furthermore, by extending the analysis to human colon tumor tissue, we provided proof of principle that the transcription site profile of cells varies among individual tumors and may be used to predict patient response to 5-FU. This study will be followed up by a more extensive clinical test of the approach to determine the predictive value of such data.

We suggest transcription site analysis of cells *in situ* will be more incisive than expression analysis or profiling in subclassifying tumors with regard to important clinical phenotypes, such as chemotherapeutic response, as reported here, or invasive or metastatic potential. Such information is essential for maximizing the efficacy of therapeutic treatment, and for personalizing care for patients.

Disclosure of Potential Conflicts of Interest

L.H. Augenlicht: consultant/advisory board, Pittsburgh Cancer Center, Arizona Cancer Center, and Valley Hospital, New Jersey. R.H. Singer: consultant/advisory board, Aureon Laboratories. The other authors disclosed no potential conflicts of interest.

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