Effectiveness of Gene Expression Profiling for Response Prediction of Rectal Adenocarcinomas to Preoperative Chemoradiotherapy

B. Michael Ghadimi, **Marian Grade**, **Michael J. Difilippantonio**, **Sudhir Varma**, **Richard Simon**, **Cristina Montagna**, **Laszlo Füzesi**, **Claus Langer**, **Heinz Becker**, **Torsten Liersch**, and **Thomas Ried**

Departments of General Surgery and Pathology, University Medical Center Göttingen, Göttingen, Germany

Genetics Branch, Center for Cancer Research, and Biometrics Research Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD

Abstract

Purpose—There is a wide spectrum of tumor responsiveness of rectal adenocarcinomas to preoperative chemoradiotherapy ranging from complete response to complete resistance. This study aimed to investigate whether parallel gene expression profiling of the primary tumor can contribute to stratification of patients into groups of responders or nonresponders.

Patients and Methods—Pretherapeutic biopsies from 30 locally advanced rectal carcinomas were analyzed for gene expression signatures using microarrays. All patients were participants of a phase III clinical trial (CAO/ARO/AIO-94, German Rectal Cancer Trial) and were randomized to receive a preoperative combined-modality therapy including fluorouracil and radiation. Class comparison was used to identify a set of genes that were differentially expressed between responders and nonresponders as measured by T level downsizing and histopathologic tumor regression grading.

Results—In an initial set of 23 patients, responders and nonresponders showed significantly different expression levels for 54 genes ($P < .001$). The ability to predict response to therapy using gene expression profiles was rigorously evaluated using leave-one-out cross-validation. Tumor behavior was correctly predicted in 83% of patients ($P = .02$). Sensitivity (correct prediction of response) was 78%, and specificity (correct prediction of nonresponse) was 86%, with a positive and negative predictive value of 78% and 86%, respectively.

Conclusion—Our results suggest that pretherapeutic gene expression profiling may assist in response prediction of rectal adenocarcinomas to preoperative chemoradiotherapy. The

Authors' Disclosures of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

implementation of gene expression profiles for treatment stratification and clinical management of cancer patients requires validation in large, independent studies, which are now warranted.

INTRODUCTION

Surgery is the primary treatment for rectal adenocarcinomas. In locally advanced stages of the disease, surgery is supported by radiation or combined-modality therapy (chemotherapy and radiation) to reduce the risk of local recurrence. Chemoradiotherapy can be administered before surgery or after tumor resection; however, the timing is still a matter of controversy.[1-3] Preoperative regimens might be particularly attractive for the following reasons: (1) a priori not curatively resectable tumors can possibly be downsized to achieve the undisputed benefit of tumor cell–free surgical margins (R0 resection); (2) preoperative treatment has the potential to reduce tumor burden and, therefore, might increase the possibility for sphincter preservation; and (3) postoperative clinical complications preclude subsequent adjuvant chemoradiotherapy.

However, the response of individual tumors to adjuvant therapies, either pre- or postoperatively, is not uniform. This poses a considerable clinical dilemma because patients with a priori resistant tumors could be spared exposure to radiation or DNA-damaging drugs, treatments that are associated with substantial adverse effects, and surgery could be scheduled without delay. Alternatively, different adjuvant treatment modalities, including additional chemotherapeutics, could be pursued. Therefore, it would be of significant clinical relevance to identify predictive markers of cancer response to chemotherapy or combined-modality therapy. Accordingly, numerous groups have used targeted approaches to correlate expression levels of candidate genes in response to different chemotherapeutic drugs, radiation, and combinations of chemotherapy and radiation (recently reviewed by Pasche et al[4] and Adlard et al[5]). For instance, thymidylate synthase (TS) and other fluorouracil (FU)-associated enzymes, such as thymidylate phosphorylase and dihydropyrimidine dehydrogenase, have been analyzed with respect to local recurrence and development of metastases of colorectal carcinomas after postoperative FU chemotherapy. High levels of TS are associated with decreased disease-free survival and poorer overall outcome in colorectal carcinomas.[6-10]

In contrast to colon carcinomas for which adjuvant treatment includes exclusively chemotherapy, preoperative treatment of locally advanced rectal carcinomas can be based on either radiation alone or combination therapy (chemotherapy and radiation). Therefore, measuring expression levels of TS may not be sufficient for response prediction in rectal carcinomas treated with combined-modality therapy. This prompted the exploration of additional or alternative markers, such as p53, p27, p21, bcl2, Ki-67, PCNA, MSH2, and surviving for response prediction.[11-26] However, the predictive value of at least some of these markers remains controversial.[11-13,22]

The value of gene expression profiling based on microarray technologies for the prediction of drug response was tested in several model systems, including the NCI-60 cancer cell line panel,[27,28] in tumor xenografts,[29] in 30 established colorectal cancer cell lines,[30] in primary tumors,[31] and during treatment.[32] Gene expression profiling also showed value in prediction

of recurrence in Dukes' B colon carcinomas.[33] The results of these studies provide evidence that, at least for some tumors and a subset of drugs, pretherapeutic gene expression profiles might predict treatment response. This prompted us to explore whether gene expression profiles of primary rectal carcinomas could offer insight into specific transcriptional patterns that determine response to preoperative chemoradiotherapy. To address this question, we ascertained, under strictly standardized conditions, diagnostic biopsies from 30 patients with locally advanced rectal carcinomas (determined by rectal ultrasound as uT3 and uT4) who are participants of a prospective randomized phase III trial (CAO/ARO/AIO-94, German Rectal Cancer Trial).[34] Global gene expression profiles were evaluated using microarrays. Class-comparison statistics were used to correlate the expression results with tumor downsizing after preoperative chemoradiotherapy and with morphologic tumor regression according to a grading system[35,36] that was used in the German Rectal Cancer Trial.[37] The robustness of the analysis was rigorously assessed using an established statistical method (ie, leave-one-out cross-validation [LOOCV]) and validated on an independent, oligonucleotide-based microarray platform.

PATIENTS AND METHODS

Patients, Samples, and Treatment

All patients are participants in a multicenter, randomized prospective phase III clinical trial (CAO/ARO/AIO-94, German Rectal Cancer Trial) aimed at determining the relative benefits of preoperative versus postoperative chemoradiotherapy.[34] Preoperative chemoradiotherapy, the histologic diagnosis, and surgical resection of carcinomas were standardized as part of the clinical trial. For the study here, we prospectively collected biopsies from 30 patients assigned to the preoperative treatment arm adhering to the guidelines set by the local ethical review board. Pretherapeutic staging was performed, including complete medical history and physical evaluation, digital rectal examination, endorectal ultrasound, rigid rectoscopy with two tumor biopsies, colonoscopy, abdominal ultrasound, abdominal and pelvic computed tomography, and chest x-ray. Endoscopic ultrasound was performed by two experienced surgeons (B.M.G. and C.L.), and staging was based on the degree of tumor infiltration through the rectal wall (T level) according to standard criteria.[38–40] In previous studies, we showed that T level assessment as performed by pretherapeutic rectal ultrasound (uT) correlates well with the histomorphologic diagnosis of resected tumor specimens.[38] Only patients with uT level 3 (n=29) and uT level 4 (n=1) carcinomas located within 16 cm from the anocutaneous verge were included. Two pretherapeutic biopsies were taken from representative adjacent areas of the tumors, one of which was examined by a pathologist (L.F.). The second biopsy was used for RNA extraction.

All 30 patients subsequently received a total dose of 50.4 Gy of radiation (single dose of 1.8 Gy, delivered in 28 fractions using a three- or four-field box technique with individually shaped portals) accompanied by FU (1,000 mg/m^2/d on days 1 through 5 and days 28 through 33) as a 120-hour continuous intravenous application.[34]

Standardized surgery was performed, including total mesorectal excision,[41] after an interval of approximately 6 weeks after chemoradiotherapy. The histologic work-up of surgical

specimens was performed according to International Union Against Cancer (UICC) standards.[42] The experimental design is summarized in Figure 1, and the clinical data are listed in Table 1. Additionally, three matched pairs of preoperative biopsies and resected tumor specimens from patients of the adjuvant treatment arm were analyzed to establish the degree of similarity between the biopsy and resected specimen.

Response Classification

Response classification of primary tumors to preoperative therapy is problematic. This is reflected by the fact that several classifications exist, two of which we have used here. First, response was evaluated as downsizing of the primary rectal cancer by comparing the uT category (determined by rectal ultrasound) with the histopathology after completion of preoperative chemoradiotherapy. This response classification is referred to as T level downsizing and was defined as the reduction of tumor infiltration by at least one T level. We thought that using an established response classification system was an acceptable intermediate end point. Once 5-year survival rates become available, we will establish correlations between the results presented here and survival. Furthermore, T level downsizing is an important clinical parameter with direct impact on patient management and prognosis with respect to sphincter preservation and R0 resections rates.

Second, histopathologic tumor regression was based on a semiquantitative classification system as proposed earlier.[35,36] As previously reported,[22] tumors were considered responsive when assigned to the regression grades 3 and 4 because these grades are defined by the almost complete (grade 3) or complete (grade 4) absence of remaining tumor cells. All other grades were classified as nonresponsive. The clinical data and tumor response classifications are listed in Table 1.

RNA Isolation and Expression Profiling

After rectal ultrasound, tumor biopsies were ascertained and stored immediately in RNAlater (Ambion, Austin, TX). Tumor material was in the range of 5 mg to 80 mg. RNA extraction was performed using TRIZOL (Invitrogen, Carlsbad, CA) following standard procedures. High-quality RNA could be successfully extracted from all samples. To generate enough RNA for repeat hybridizations, mRNA was amplified using the RiboAmp RNA amplification kit (Arcturus, Mountain View, CA) for the cDNA arrays and the Amino Allyl MessageAmp aRNA Kit (Ambion) for the oligonucleotide arrays, which resulted in antisense mRNA amounts that averaged 50 μg. RNA was labeled indirectly incorporating 5-(3-Aminoallyl)-2′-deoxy-uridine-5′-triphosphate, followed by chemical coupling of Cy3 (Amersham, Piscataway, NJ). Control cRNA was generated by amplification of a reference mRNA pool (Stratagene, La Jolla, CA) and labeled using Cy5 (Amersham). RNA quantification and labeling efficiency was determined using the Nanodrop quantification device (Nanodrop, Rockland, DE).

Expression profiling was carried out on the National Cancer Institute cDNA (9,984 features) and oligonucleotide (22,231 features) arrays as follows: 5.0 μg of Cy3-labeled test cDNA/cRNA and 5.0 μg of Cy5-labeled control cDNA/cRNA were hybridized at 42°C overnight in specifically designed hybridization cassettes (TeleChem International, Sunnyvale, CA).

After hybridization, slides were washed and scanned on an Axon scanner using GenePixPro (3.0) software (Axon Instruments, Union City, CA). Spot quality was assessed according to criteria in GenePixPro (3.0) software. Background subtraction and normalization was performed on extraction of the data from the Center for Information Technology/National Institutes of Health microarray database, mAdb (http://nciarray.nci.nih.gov/). Spots with a size of less than 10 μm or an intensity less than 100 in both the red and green channels were eliminated, as were genes with more than 50% of missing data. This a priori filtering to remove genes with unreliable signals resulted in a final tally of 9,059 genes on the cDNA arrays and 12,291 genes on the oligonucleotide arrays. An initial set of 23 tumors was hybridized to cDNA arrays. To establish the reproducibility on different platforms, 10 of these 23 samples and an additional set of seven tumors were hybridized to oligonucleotide arrays.

Statistical Analysis

The statistical analyses were performed using the BRB Array-Tools package for microarray analysis developed at the Biometric Research Branch of the National Cancer Institute (Bethesda, MD)[43] and MATLAB from The Mathworks (Natick, MA). The two technical replicate expression profiles of the same tumor specimen were highly consistent (correlation coefficient > 0.95) and were averaged for analysis. A class-comparison analysis was applied to determine which genes were differentially expressed between the two classes. For this, we used a two-sample *t* test with a randomized variance model.[44] The randomized variance model assumes that the variance of the expression of each gene is randomly drawn from an inverse-gamma distribution and enables sharing of variance information among genes without assuming that all genes have the same variance. We used a stringent statistical significance threshold of *P*<.001 for the identification of genes differentially expressed between responders and nonresponders. This controls for the number of genes tested. Tests were performed for 9,059 genes, which passed the filtering criteria, and the expected number of false-positive genes was thus nine. Because 54 genes were found to be significant at *P*<.001 for T level downsizing, the false discovery rate was 16.67%.

We applied diagonal linear discriminant analysis[45] to predict response. The genes that are differentially expressed at a *P*<.001 level were used as predictive features in the classifiers. Complete LOOCV was used to estimate the prediction accuracy for each classification method. With LOOCV, one sample is left out, and the remaining samples are used to build a classifier, which is then used to classify the left-out sample. Both feature selection and classifier design was repeated each time a different sample was left out, thus this analysis was repeated 23 times. The fraction of samples that are classified correctly is an estimate of the classification accuracy. LOOCV is a more efficient method of estimating prediction error than the split-sample approach of dividing the data once into a training set and a validation set. LOOCV provides unbiased estimates of prediction error that are as valid as those for the split-sample approach as long as the entire model building process is repeated for each leave-one-out training set.[46,47]

The significance of the classification results is calculated by permuting the class labels of the samples and then finding the fraction of times this relabeling resulted in higher LOOCV

classification accuracy. We permuted 10,000 times, which resulted in $P = .021$. The method takes into account the sample size and is suitable for small sample numbers.[48]

Ten samples out of the 23 hybridized on cDNA were also hybridized to oligonucleotide arrays. This set of 10 was used to calculate a normalization to make the oligonucleotide data comparable to the cDNA data. We found that there is a consistent gene-specific bias between the oligonucleotide and cDNA measurements of gene expression (ie, for any particular gene, there is a difference in the log-expression ratios measured using cDNA and oligonucleotide that is consistent across samples, but depends on the specific gene). If $O(g,s)$ denotes the gene expression measured using oligonucleotide arrays for gene g (there are 6,939 common genes between the cDNA and oligonucleotide arrays) and sample s, and $C(g,s)$ denotes the corresponding measurement using cDNA arrays, then:

$$C(g,s) = O(g,s) + B(g) + \eta$$

where $B(g)$ is the gene-specific bias for gene g, and η is random noise with zero mean. This implies that the change in platform from cDNA array to oligonucleotide array introduces an additive gene-specific effect in the measured log ratios. We obtained an estimate of $B(g)$ for each gene as follows:

$$\hat{B}(g) = \overline{C}(g) - \overline{O}(g)$$

where $\overline{C}(g)$ and $\overline{O}(g)$ are the means of $C(g,s)$ and $O(g,s)$ over the 10 samples hybridized to both platforms.

A set of seven independent tumors was hybridized to oligonucleotide arrays only. The estimate for the bias $\hat{B}(g)$ was added to the oligonucleotide data to make it possible to use the classifier developed on cDNA data to be used to classify the oligonucleotide data.

The list of genes with significantly different expression ($P<.001$) established in the cDNA array set of 23 patients and the diagonal linear discriminant classifier based on these samples were then used to predict treatment response for the seven patients in the oligonucleotide set. The summary of the data analysis scheme is presented in Figure 2.

RESULTS

Response Classification of Rectal Adenocarcinomas to Preoperative Chemoradiotherapy

To identify molecular signatures of responsiveness to preoperative chemoradiotherapy, we analyzed gene expression profiles from 30 rectal adenocarcinoma biopsies from participants of a randomized clinical trial (CAO/ARO/AIO-94, German Rectal Cancer Trial). The flow of sample collection, clinical diagnosis, and experimental design is depicted in Figure 1. Before biopsy sampling, initial tumor staging was performed by rectal ultrasound. The majority of patients were diagnosed with cUICC III (n = 22) carcinomas, whereas the remaining eight patients were diagnosed with cUICC II carcinomas. In all instances, duplet biopsies were ascertained and submitted to either histopathologic evaluation or expression

profiling. Preoperative chemoradiotherapy, the histologic diagnosis, and surgical resection of carcinomas were standardized as part of the clinical trial. Histopathologic evaluation of the tumor biopsies revealed that all of the rectal cancers included in our analysis were adenocarcinomas. The clinical data and response classifications are listed in Table 1. Response classification was based on T level downsizing and histopathologic regression grading.[36,49] With respect to T level downsizing, only patients who showed at least one T level reduction were considered responsive (patients 1 to 9 from the cDNA array set and patients 24, 25, 27, 28, and 30 from the oligonucleotide array set). Thus, 14 of the prospectively collected samples were from patients who were later classified as responders, and 16 patients showed no T level response. Regarding the regression grading, patients with grades 3 and 4 were considered responders (patients 1 to 8 and 10 to 13 from the cDNA microarray set and patients 24, 27, and 28 from the oligonucleotide array set), which left us with an equal number of responders and nonresponders.

Gene expression profiling: class comparison—Gene expression profiles of the first 23 tumor biopsies were established using cDNA arrays (9,984 features). For each biopsy, technical repeat hybridizations were performed, and the coefficient of correlation was determined to be more than 95%. This excellent correlation allowed us to average the replicates for each patient before evaluation. Using class-comparison analysis, we identified a list of 54 genes that were differentially expressed at significance levels ($P < .001$) between responsive and nonresponsive tumors based on T level down-sizing (Table 2). In the group of responders, 41 genes showed lower expression relative to the group of nonresponders. The list of genes includes *calmin*, *kinectin 1*, *copine III*, *villin-like*, *motilin*, *cdc42* (*binding protein A*), *myosin IA*, *cyclin T1*, *interleukin 12A*, *SMC1*, *platelet derived growth factor C*, and a number of genes that encode proteins involved in signaling and membrane transport and proteins with varying enzymatic properties (thiolase, lipase, peptidase and protease activity). The results of a hierarchical cluster analysis of the 54 genes are presented pictorially in Figure 3, along with the gene annotation and fold change between the groups. To corroborate the significance of finding these genes in our analysis, we have also determined that the likelihood that 54 genes would be significantly ($P < .001$) changed by chance to be exceedingly low ($P = .008$).

Using the regression grading classification, only four genes (*REGL*, *ACVR2B*, *SMARCC1*, and *ZNF134*) and one expressed sequence tag (EST) were differentially expressed ($P < .001$) between responders and nonresponders. Because the probability of five genes being differentially expressed by chance is high ($P=.31$), we concluded that there was no evidence for differential expression between the histopathologic response and nonresponse samples.

Gene expression profiling: class prediction—We then wished to evaluate the usefulness of gene expression profiling for response prediction. This was achieved by using an established LOOCV strategy. Classification results for T level downsizing were derived using a diagonal linear discriminant[45] analysis. Four patients were incorrectly classified; two belonged to the nonresponse group but were classified as responders (patients 15 and 21), and two belonged to the response group but were classified as nonresponders (patients 1 and 2). Therefore, the estimate of the class prediction accuracy using LOOCV results in 83%

correct assignment as to either responders or nonresponders (P=.02). This P value, which indicates a low probability of obtaining this classification result by chance, was calculated using a permutation method that takes the sample size into account (see Patients and Methods). Table 3 shows the correlation between class prediction and T level downsizing. The sensitivity of the test (percentage of responders who were predicted correctly as responders) was 78%. The specificity (ie, patients who were correctly classified as nonresponders to preoperative chemoradiotherapy) was 86%. The positive predictive value (percentage of patients classified as responders who were true responders) was 78%, and the negative predictive value (percentage of patients classified as nonresponders who were true nonresponders) was 86%. Because only five genes were differentially expressed when using the histopathologic regression grading classification, no further LOOCV was attempted.

We then wanted to explore whether gene expression profiling for response prediction was independent of the microarray platform used. Therefore, we hybridized a newly obtained set of seven tumors to oligonucleotide arrays. Thirty-nine of the 54 genes identified as statistically significant using the cDNA arrays had corresponding features on the oligonucleotide arrays. Thus, our diagonal linear discriminant classifier was trained using the expression of these 39 genes from the 23 tumors hybridized to cDNA arrays. This cDNA classifier was able to correctly predict the response of six out of seven patients (86%; Table 3) and, thus, attests to the robustness of microarray profiling and to the biologic relevance of the genes identified here. The flow of the different statistical tools used for class comparison, class prediction, and cross-platform assessment is summarized in Figure 2.

DISCUSSION

A recently published report on the results of a randomized phase III clinical trial (CAO/ARO/AIO-94, German Rectal Cancer Trial) provided convincing evidence that preoperative chemoradiotherapy of rectal adenocarcinomas reduces local recurrence (6% after 5 years) when compared with postoperative (13% after 5 years) multimodality treatment.[37] Given these results, neoadjuvant therapy has now been adapted as the therapy of choice by the participating clinics. However, not all tumors respond uniformly, and despite promising results, a priori resistance to chemoradiotherapy poses a thorny problem because patients with nonresponsive tumors could either be spared from possible side effects of cytotoxic treatment and radiation or be subjected to alternative treatment modalities.[50,51] Therefore, developing tools for response prediction has become exceedingly important. Thus, we collected, prospectively, diagnostic biopsies from 30 patients from the German Rectal Cancer Trial with locally advanced rectal adenocarcinomas.

We acknowledge that there is a trade-off between sample size and the accrual of tumor specimens under strictly standardized diagnostic and therapeutic procedures. For the pilot study here, our patients were limited to those who were recruited to the German Rectal Cancer Trial and who were treated at only one of the participating clinical centers (because sample collection, surgery, and neoadjuvant treatment are identical, and post-therapy follow-up is significantly simplified). Our sample size is taken into account when calculating the P value for the classification accuracy. Although we have begun to test the robustness of our approach by evaluating the performance of the classifier on a different array platform, the

ultimate test of the robustness and clinical applicability would require multicenter sample accrual and processing. Therefore, we have initiated such a study.

Using T level downsizing, the results of our gene expression analyses resulted in a classifier with high accuracy (83%), with only four of 23 patients incorrectly classified when using LOOCV. A similar accuracy (86%) was achieved with the independent set of seven patients, despite the difference in microarray platform; thus, utilization of gene expression profiling by microarray analysis seems robust enough to be independent of the particular platform used. Our inability to achieve higher accuracy could be due to several reasons, including tumor heterogeneity or the possibility that contamination of these particular biopsies with either normal rectal epithelium or adenomatous or stromal tissue could have partially obscured the detection of gene expression profiles more specific to rectal tumor cells. The latter does not seem likely, however, because we have established on frozen sections that the tumor cell content in the biopsies was greater than 90%. Incorrect tumor staging based on pretherapeutic rectal ultrasound could also result in incorrect classification of T level response. Finally, genes contributing to tumor response and, thus, useful for classification might not be represented on the arrays used in our study, or responsiveness of these tumors depends on post-translational modifications, which can obviously not be detected by gene expression analysis on nucleotide arrays.

The 54 genes that had expression levels that were most significantly changed ($P < .001$) represent members of several cellular pathways and map to multiple different chromosomes. Of particular interest are genes that encode proteins involved in DNA damage repair pathways, such as *SMC1*, which is involved in the S-phase checkpoint mediated by ATM.[52,53] A number of genes were involved in microtubule organization. For instance, *calmin* has homology to *calponin* and *dystrophin*.[54] *Cdc42BPA* is a member of the *Rho GTPase* subfamily and triggers microtubule reorganization and cytoskeletal remodeling through GSK-3 and APC, which are two proteins involved in Wnt signaling.[55,56] *Filamin B* is an integrator of cell mechanics and signaling and acts in the *Rho* signaling apparatus,[57] *villin* proteins are involved in actin metabolism,[58] and *kinectin 1* is a binding partner of *kinesin* and belongs to a class of molecular motors involved in mitoses, axoplasmic transport, and secretion.[59] The preponderance of genes involved in microtubule organization cannot be explained by relative abundance of members of such gene families alone (http://nciarray.nci.nih.gov/).

Using the histopathologic regression grading, we only identified five genes that were differentially expressed ($P<.001$) between the two groups of responders and nonresponders. Because the probability of this number of genes being differentially expressed by chance was high ($P = .31$), we abstained from further statistical analyses. A possible explanation could be our definition of response because we defined grade 3 and 4 patients as responders according to previously published studies.[22] However, it might be more reasonable to divide the groups into complete responders (regression grade 4) and nonresponders or partial responders (grades 0 to 3). This was not possible in our study because of the unequal distribution of biopsies (only three patients were assigned response grade 4).

Several key proteins involved in the repair of radiation-induced DNA damage and enzymes involved in thymidine metabolism have been analyzed in the past regarding their potential to predict tumor response in individual patients.[4,5] For instance, the expression levels of TS are usually inversely correlated with response to adjuvant FU treatment. However, the potential of TS expression levels to predict response to preoperative combined-modality therapy remains less conclusive. One possible explanation could be that many more studies were conducted regarding response prediction to postoperative chemotherapy than to preoperative therapy. Second, one could surmise that radiation, rather than chemotherapy, constitutes the main treatment component of preoperative chemoradiotherapy. A number of groups established correlations of expression levels of the tumor suppressor genes *p53*, *p21*, and *p27* as well as *bcl2*, *Ki-67*, *MSH2*, *survivin*, *TS*, and *PCNA* with therapy response.[11–26] However, conflicting conclusions were reached as to the predictive power of any one of these genes, some of which were included on our arrays. These discrepancies can be a result of different study designs, different pretherapeutic staging classifications, nonstandardized treatments, different definitions of response, and different methodologies for determination of gene expression levels. The expression levels of these genes were not significantly different ($P > .1$) in groups of responders or nonresponders in our study.

In this study, we demonstrate that analysis of even a limited subset of patients from the randomized German Rectal Cancer Trial[37] by gene expression profiling may contribute to the clinical stratification of responders and nonresponders. We have validated the robustness of our approach using different array platforms; however, we feel that, from a clinical perspective, our results should be confirmed with larger patient cohorts.

Acknowledgments

References

1. Nelson H, Sargent DJ. Refining multimodal therapy for rectal cancer. N Engl J Med. 2001; 345:690–692. [PubMed: 11547725]

2. Minsky BD. Adjuvant therapy for rectal cancer: The transatlantic view. Colorectal Dis. 2003; 5:416–422. [PubMed: 12925072]

3. Sauer R. Adjuvant and neoadjuvant radiotherapy and concurrent radiochemotherapy for rectal cancer. Pathol Oncol Res. 2002; 8:7–17. [PubMed: 11994757]

4. Pasche B, Mulcahy M, Benson AB 3rd. Molecular markers in prognosis of colorectal cancer and prediction of response to treatment. Best Pract Res Clin Gastroenterol. 2002; 16:331–345. [PubMed: 11969242]

5. Adlard JW, Richman SD, Seymour MT, et al. Prediction of the response of colorectal cancer to systemic therapy. Lancet Oncol. 2002; 3:75–82. [PubMed: 11902527]

6. Popat S, Matakidou A, Houlston RS. Thymidylate synthase expression and prognosis in colorectal cancer: A systematic review and meta-analysis. J Clin Oncol. 2004; 22:529–536. [PubMed: 14752076]

7. Allegra CJ, Paik S, Colangelo LH, et al. Prognostic value of thymidylate synthase, Ki-67, and p53 in patients with Dukes' B and C colon cancer: A National Cancer Institute-National Surgical Adjuvant Breast and Bowel Project collaborative study. J Clin Oncol. 2003; 21:241–250. [PubMed: 12525515]

8. Aschele C, Debernardis D, Casazza S, et al. Immunohistochemical quantitation of thymidylate synthase expression in colorectal cancer metastases predicts for clinical outcome to fluorouracil-based chemotherapy. J Clin Oncol. 1999; 17:1760–1770. [PubMed: 10561213]

9. Johnston PG, Lenz HJ, Leichman CG, et al. Thymidylate synthase gene and protein expression correlate and are associated with response to 5-fluorouracil in human colorectal and gastric tumors. Cancer Res. 1995; 55:1407–1412. [PubMed: 7882343]

10. Salonga D, Danenberg KD, Johnson M, et al. Colorectal tumors responding to 5-fluorouracil have low gene expression levels of dihydropyrimidine dehydrogenase, thymidylate synthase, and thymidine phosphorylase. Clin Cancer Res. 2000; 6:1322–1327. [PubMed: 10778957]

11. Okonkwo A, Musunuri S, Talamonti M, et al. Molecular markers and prediction of response to chemoradiation in rectal cancer. Oncol Rep. 2001; 8:497–500. [PubMed: 11295069]

12. Saw RP, Morgan M, Koorey D, et al. p53, deleted in colorectal cancer gene, and thymidylate synthase as predictors of histopathologic response and survival in low, locally advanced rectal cancer treated with preoperative adjuvant therapy. Dis Colon Rectum. 2003; 46:192–202. [PubMed: 12576893]

13. Spitz FR, Giacco GG, Hess K, et al. p53 immunohistochemical staining predicts residual disease after chemoradiation in patients with high-risk rectal cancer. Clin Cancer Res. 1997; 3:1685–1690. [PubMed: 9815551]

14. Rodel F, Hoffmann J, Grabenbauer GG, et al. High survivin expression is associated with reduced apoptosis in rectal cancer and may predict disease-free survival after preoperative radiochemotherapy and surgical resection. Strahlenther Onkol. 2002; 178:426–435. [PubMed: 12240548]

15. Luna-Perez P, Arriola EL, Cuadra Y, et al. p53 protein overexpression and response to induction chemoradiation therapy in patients with locally advanced rectal adenocarcinoma. Ann Surg Oncol. 1998; 5:203–208. [PubMed: 9607618]

16. Elsaleh H, Robbins P, Joseph D, et al. Can p53 alterations be used to predict tumour response to pre-operative chemo-radiotherapy in locally advanced rectal cancer? Radiother Oncol. 2000; 56:239–244. [PubMed: 10927144]

17. Scott N, Hale A, Deakin M, et al. A histopathological assessment of the response of rectal adenocarcinoma to combination chemoradiotherapy: Relationship to apoptotic activity, p53 and bcl-2 expression. Eur J Surg Oncol. 1998; 24:169–173. [PubMed: 9630854]

18. Esposito G, Pucciarelli S, Alaggio R, et al. P27kip1 expression is associated with tumor response to preoperative chemoradiotherapy in rectal cancer. Ann Surg Oncol. 2001; 8:311–318. [PubMed: 11352304]

19. Gunther K, Dimmler A, Rodel F, et al. P27 does not predict histopathological response to radiochemotherapy in rectal cancer. J Surg Res. 2003; 113:179–188. [PubMed: 12957127]

20. Rau B, Sturm I, Lage H, et al. Dynamic expression profile of p21WAF1/CIP1 and Ki-67 predicts survival in rectal carcinoma treated with preoperative radiochemotherapy. J Clin Oncol. 2003; 21:3391–3401. [PubMed: 12885834]

21. Tannapfel A, Nusslein S, Fietkau R, et al. Apoptosis, proliferation, bax, bcl-2 and p53 status prior to and after preoperative radiochemotherapy for locally advanced rectal cancer. Int J Radiat Oncol Biol Phys. 1998; 41:585–591. [PubMed: 9635706]

22. Rodel C, Grabenbauer GG, Papadopoulos T, et al. Apoptosis as a cellular predictor for histopathologic response to neoadjuvant radiochemotherapy in patients with rectal cancer. Int J Radiat Oncol Biol Phys. 2002; 52:294–303. [PubMed: 11872273]

23. Kim NK, Park JK, Lee KY, et al. p53, BCL-2, and Ki-67 expression according to tumor response after concurrent chemoradiotherapy for advanced rectal cancer. Ann Surg Oncol. 2001; 8:418–424. [PubMed: 11407516]

24. Willett CG, Warland G, Cheek R, et al. Proliferating cell nuclear antigen and mitotic activity in rectal cancer: Predictor of response to preoperative irradiation. J Clin Oncol. 1994; 12:679–682. [PubMed: 7908689]

25. Willett CG, Hagan M, Daley W, et al. Changes in tumor proliferation of rectal cancer induced by preoperative 5-fluorouracil and irradiation. Dis Colon Rectum. 1998; 41:62–67. [PubMed: 9510312]

26. Qiu H, Sirivongs P, Rothenberger M, et al. Molecular prognostic factors in rectal cancer treated by radiation and surgery. Dis Colon Rectum. 2000; 43:451–459. [PubMed: 10789738]

27. Staunton JE, Slonim DK, Coller HA, et al. Chemosensitivity prediction by transcriptional profiling. Proc Natl Acad Sci U S A. 2001; 98:10787–10792. [PubMed: 11553813]

28. Scherf U, Ross DT, Waltham M, et al. A gene expression database for the molecular pharmacology of cancer. Nat Genet. 2000; 24:236–244. [PubMed: 10700175]

29. Zembutsu H, Ohnishi Y, Tsunoda T, et al. Genome-wide cDNA microarray screening to correlate gene expression profiles with sensitivity of 85 human cancer xenografts to anticancer drugs. Cancer Res. 2002; 62:518–527. [PubMed: 11809704]

30. Mariadason JM, Arango D, Shi Q, et al. Gene expression profiling-based prediction of response of colon carcinoma cells to 5-fluorouracil and camptothecin. Cancer Res. 2003; 63:8791–8812. [PubMed: 14695196]

31. Chang JC, Wooten EC, Tsimelzon A, et al. Gene expression profiling for the prediction of therapeutic response to docetaxel in patients with breast cancer. Lancet. 2003; 362:362–369. [PubMed: 12907009]

32. Clarke PA, George ML, Easdale S, et al. Molecular pharmacology of cancer therapy in human colorectal cancer by gene expression profiling. Cancer Res. 2003; 63:6855–6863. [PubMed: 14583483]

33. Wang Y, Jatkoe T, Zhang Y, et al. Gene expression profiles and molecular markers to predict recurrence of Dukes' B colon cancer. J Clin Oncol. 2004; 22:1564–1571. [PubMed: 15051756]

34. Sauer R, Fietkau R, Wittekind C, et al. Adjuvant versus neoadjuvant radiochemotherapy for locally advanced rectal cancer: A progress report of a phase-III randomized trial (protocol CAO/ARO/AIO-94). Strahlenther Onkol. 2001; 177:173–181. [PubMed: 11370551]

35. Dworak O, Keilholz L, Hoffmann A. Pathological features of rectal cancer after preoperative radiochemotherapy. Int J Colorectal Dis. 1997; 12:19–23. [PubMed: 9112145]

36. Gavioli M, Bagni A, Piccagli I, et al. Usefulness of endorectal ultrasound after preoperative radiotherapy in rectal cancer: Comparison between sonographic and histopathologic changes. Dis Colon Rectum. 2000; 43:1075–1083. [PubMed: 10950005]

37. Sauer R, Becker H, Hohenberger W, et al. Preoperative versus postoperative chemoradiotherapy for locally advance rectal cancer. N Engl J Med. 2004; 351:1731–1740. [PubMed: 15496622]

38. Liersch T, Langer C, Jakob C, et al. Preoperative diagnostic procedures in locally advanced rectal carcinoma (> or =T3 or N+): What does endoluminal ultrasound achieve at staging and restaging (after neoadjuvant radiochemotherapy) in contrast to computed tomography? Chirurg. 2003; 74:224–234. [PubMed: 12647079]

39. Adams DR, Blatchford GJ, Lin KM, et al. Use of preoperative ultrasound staging for treatment of rectal cancer. Dis Colon Rectum. 1999; 42:159–166. [PubMed: 10211490]

40. Glaser F, Schlag P, Herfarth C. Endorectal ultrasonography for the assessment of invasion of rectal tumours and lymph node involvement. Br J Surg. 1990; 77:883–887. [PubMed: 2203508]

41. Heald RJ, Ryall RD. Recurrence and survival after total mesorectal excision for rectal cancer. Lancet. 1986; 1:1479–1482. [PubMed: 2425199]

42. Sobin, LH.; Wittekind, C. UICC: TNM Classification of Malignant Tumors. 5. New York, NY: John Wiley & Sons; 1997.

43. Simon, R.; Peng, A. BRB ArrayTools. Bethesda, MD: National Cancer Institute; 2003.

44. Wright GW, Simon RM. A random variance model for detection of differential gene expression in small microarray experiments. Bioinformatics. 2003; 19:2448–2455. [PubMed: 14668230]

45. Dudoit S, Fridlyand J, Speed TP. Comparison of discrimination methods for the classification of tumors using gene expression data. J Am Stat Assoc. 2002:77–87.

46. Ntzani EE, Ioannidis JP. Predictive ability of DNA microarrays for cancer outcomes and correlates: An empirical assessment. Lancet. 2003; 362:1439–1444. [PubMed: 14602436]

47. Simon R, Radmacher MD, Dobbin K, et al. Pitfalls in the use of DNA microarray data for diagnostic and prognostic classification. J Natl Cancer Inst. 2003; 95:14–18. [PubMed: 12509396]

48. Radmacher MD, McShane LM, Simon R. A paradigm for class prediction using gene expression profiles. J Comput Biol. 2002; 9:505–511. [PubMed: 12162889]

49. Theodoropoulos G, Wise WE, Padmanabhan A, et al. T-level downstaging and complete pathologic response after preoperative chemoradiation for advanced rectal cancer result in decreased recurrence and improved disease-free survival. Dis Colon Rectum. 2002; 45:895–903. [PubMed: 12130878]

50. Gerard JP, Chapet O, Nemoz C, et al. Preoperative concurrent chemoradiotherapy in locally advanced rectal cancer with high-dose radiation and oxaliplatin-containing regimen: The Lyon R0-04 phase II trial. J Clin Oncol. 2003; 21:1119–1124. [PubMed: 12637479]

51. Rodel C, Grabenbauer GG, Papadopoulos T, et al. Phase I/II trial of capecitabine, oxaliplatin, and radiation for rectal cancer. J Clin Oncol. 2003; 21:3098–3104. [PubMed: 12915600]

52. Yazdi PT, Wang Y, Zhao S, et al. SMC1 is a downstream effector in the ATM/NBS1 branch of the human S-phase checkpoint. Genes Dev. 2002; 16:571–582. [PubMed: 11877377]

53. Kim ST, Xu B, Kastan MB. Involvement of the cohesin protein, Smc1, in Atm-dependent and independent responses to DNA damage. Genes Dev. 2002; 16:560–570. [PubMed: 11877376]

54. Ishisaki Z, Takaishi M, Furuta I, et al. Calmin, a protein with calponin homology and transmembrane domains expressed in maturing spermatogenic cells. Genomics. 2001; 74:172–179. [PubMed: 11386753]

55. Etienne-Manneville S, Hall A. Cdc42 regulates GSK-3beta and adenomatous polyposis coli to control cell polarity. Nature. 2003; 421:753–756. [PubMed: 12610628]

56. Harwood A, Braga VM. Cdc42 & GSK-3: Signals at the crossroads. Nat Cell Biol. 2003; 5:275–277. [PubMed: 12669073]

57. Stossel TP, Condeelis J, Cooley L, et al. Filamins as integrators of cell mechanics and signalling. Nat Rev Mol Cell Biol. 2001; 2:138–145. [PubMed: 11252955]

58. Athman R, Louvard D, Robine S. The epithelial cell cytoskeleton and intracellular trafficking: III. How is villin involved in the actin cytoskeleton dynamics in intestinal cells? Am J Physiol Gastrointest Liver Physiol. 2002; 283:G496–G502. [PubMed: 12181160]

59. Karcher RL, Deacon SW, Gelfand VI. Motor-cargo interactions: The key to transport specificity. Trends Cell Biol. 2002; 12:21–27. [PubMed: 11854006]
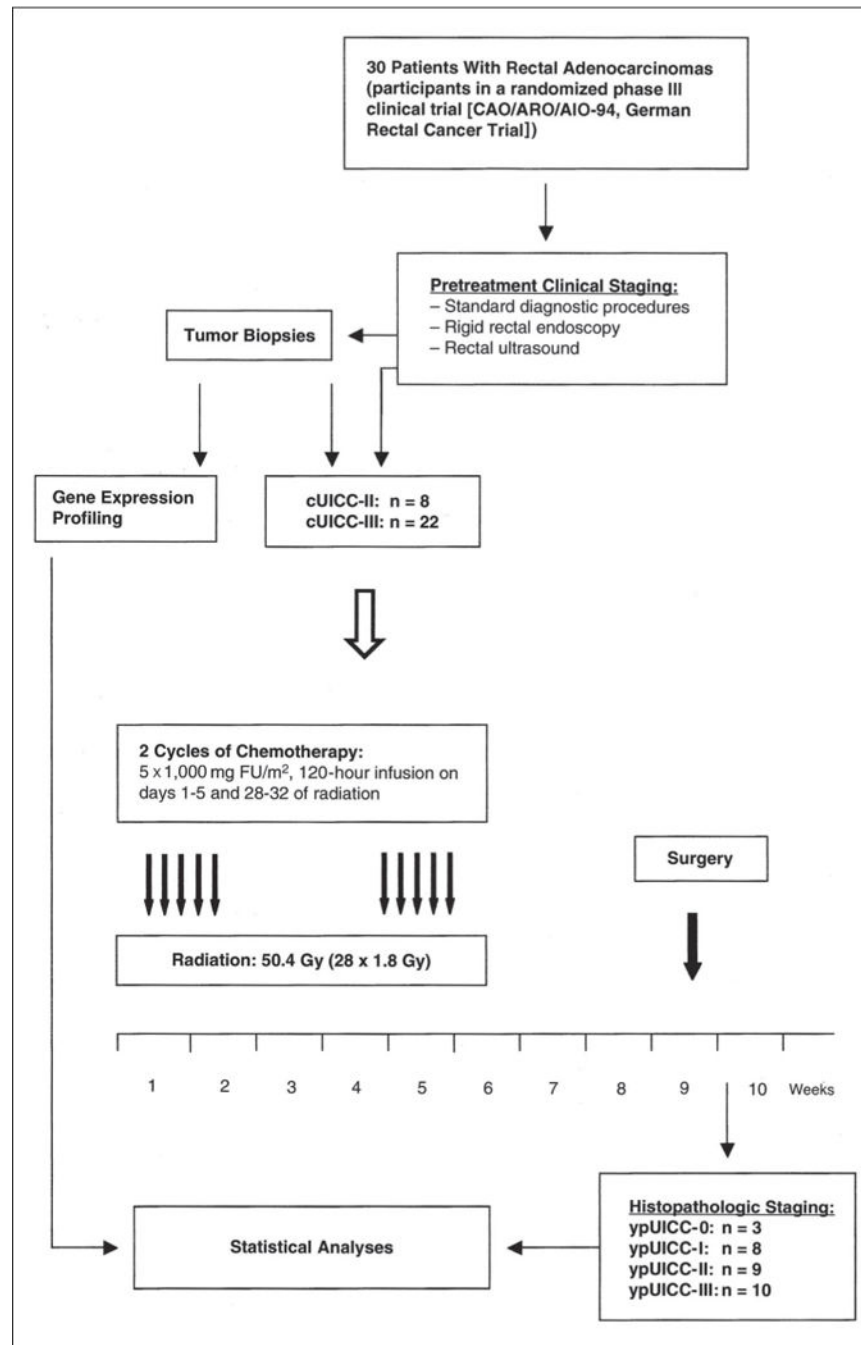
Fig 1.
Pictorial presentation of specimen accrual, clinical diagnosis, and experimental design.
cUICC refers to pretherapeutic clinical staging of tumors. ypUICC refers to histopathologic
assessment of the resected specimens after completion of preoperative therapy. UICC,
International Union Against Cancer; FU, fluorouracil.
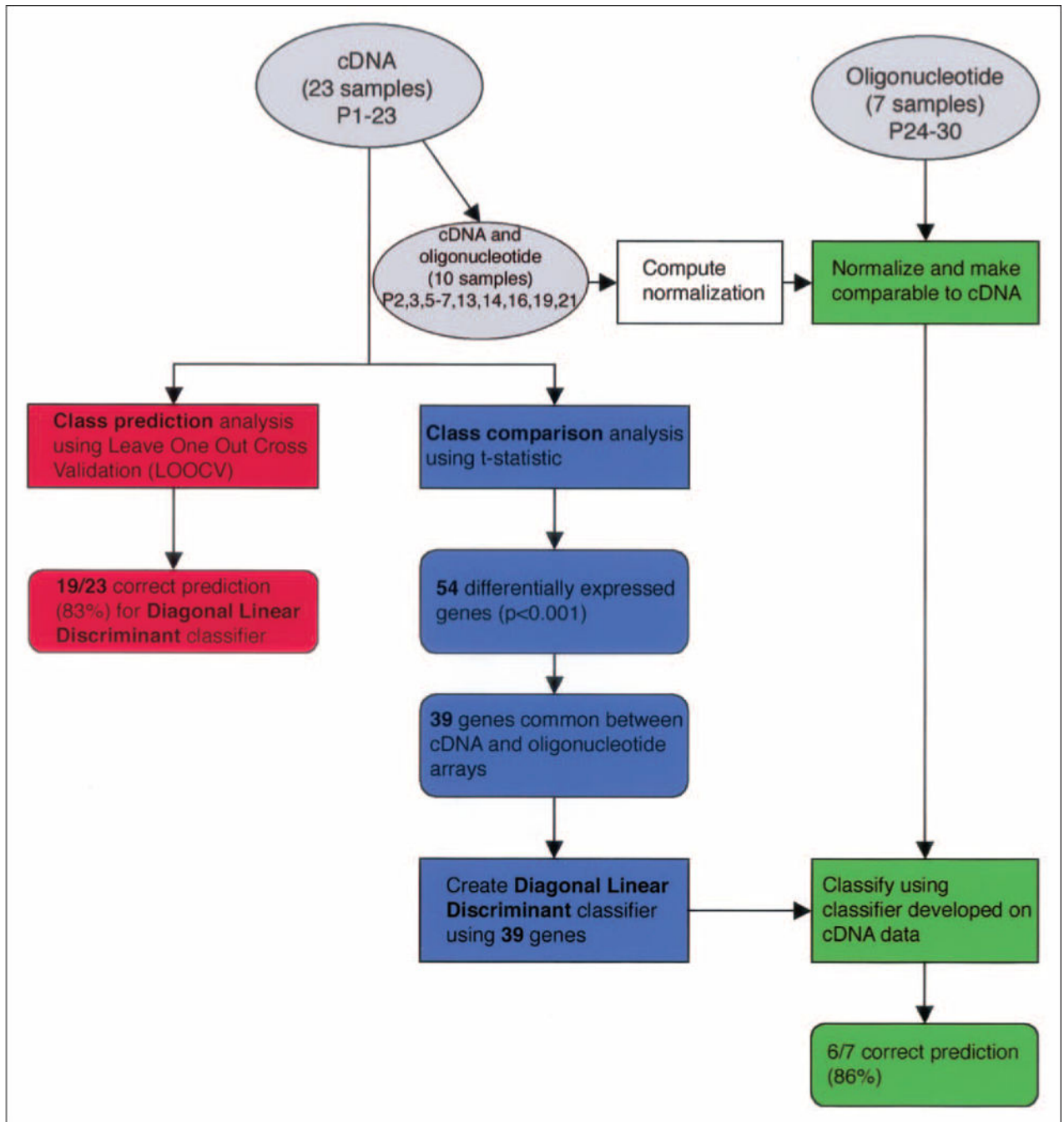
Fig 2.
Flow of statistical analyses for cDNA and oligonucleotide data. Gray ovals represent data, rectangles represent statistical procedures, and round-edged boxes indicate results. The figure shows the three main statistical analyses performed. Class prediction (red) and class comparison (blue) for cDNA data and cross-platform normalization for the classification of oligonucleotide data (green) is shown.
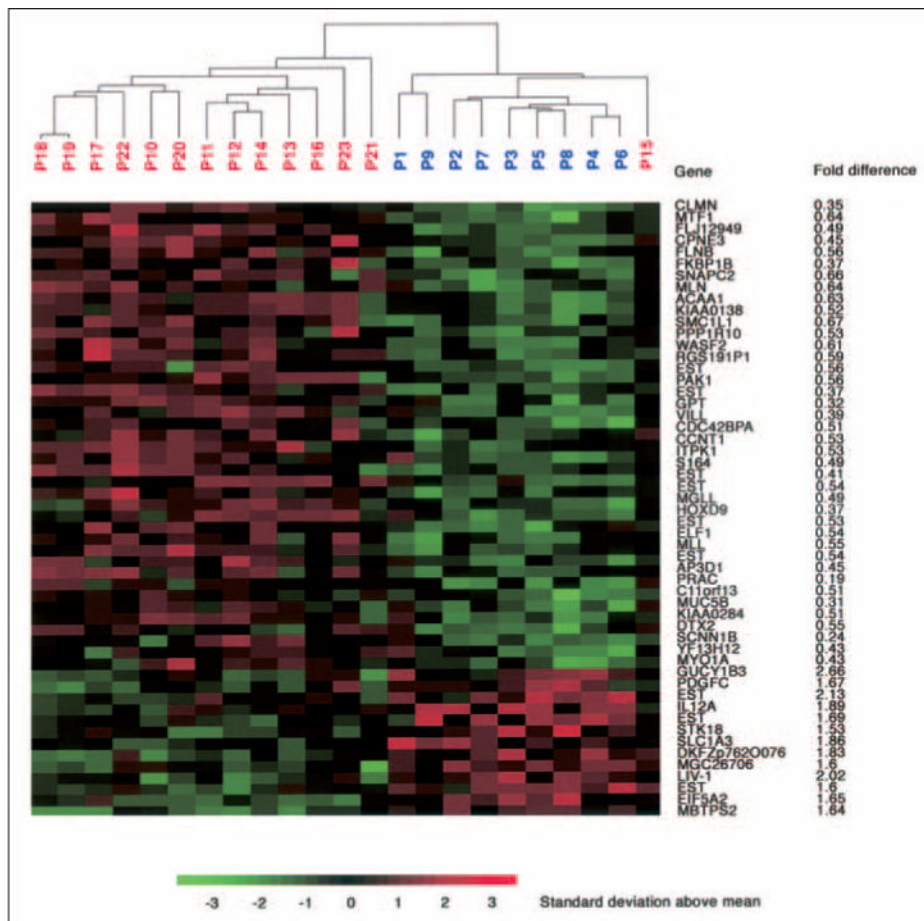
Fig 3.
Hierarchical cluster analysis of 23 patients based on the 54 most significantly changed genes (*P* < .001) when using T level downsizing. Red indicates increased expression, and green indicates decreased expression. Gene symbols and fold change between the groups are listed to the right. Values less than 1 reflect downregulation in the class of responders, whereas values more than 1 reflect upregulation. P, patient.

Table 1

Clinical Data of 30 Patients

Tumor Samples	uT	ypT	ypN	ypN Total	ypN Infiltrated	ypGrading	R	Regression Grading	cUICC	ypUICC
1	3	0	0	18	0	x	0	4	II	0
2	3	0	0	27	0	x	0	4	III	0
3	3	0	0	16	0	x	0	4	II	0
4	3	2	0	22	0	2	0	3	III	I
5	3	1	0	20	0	2	0	3	II	I
6	3	2	0	24	0	2	0	3	III	I
7	3	1	0	18	0	2	0	3	II	II
8	4	3c	1	19	3	3	0	3	III	III
9	3	2	0	16	0	3	0	2	II	I
10	3	3b	1	30	1	2	0	3	III	III
11	3	3b	0	15	0	2	0	3	III	II
12	3	3b	0	8	1	3	0	3	II	III
13	3	3b	1	27	1	2	0	3	III	III
14	3	4a	1	19	1	2	0	2	III	III
15	3	3b	0	28	0	2	0	2	III	II
16	3	3a	0	21	0	2	0	2	III	II
17	3	3b	1	19	2	3	0	2	III	III
18	3	4a	1	21	2	2	0	3	III	III
19	3	3c	0	24	0	3	0	1	III	II
20	3	3c	0	16	0	2	0	1	II	II
21	3	3c	0	17	0	2	0	1	III	II
22	3	3a	0	14	0	2	0	1	III	II
23	3	4a	1	22	1	2	0	1	III	III
24	3	2	0	16	0	2	0	3	II	I
25	3	2	0	17	0	2	0	2	III	I
26	3	3	0	20	0	2	0	2	III	II

Tumor Samples	uT	ypT	ypN	ypN Total	ypN Infiltrated	ypGrading	R	Regression Grading	cUICC	ypUICC
27	3	2	2	14	5	2	0	3	III	III
28	3	Tis	1	17	1	3	0	3	III	III
29	3	3	0	15	0	2	0	2	III	II
30	3	2	0	12	0	2	0	2	III	I

NOTE. Tumor samples 1 to 23 comprised the initial group analyzed by cDNA microarray, whereas patients 24 to 30 were used to test cross-platform robustness.

Abbreviations: uT, T level determined by rectal ultrasound before any treatment; (yp), after preoperative treatment; ypT, T level by histopathologic assessment after preoperative treatment; ypN, lymph node status by histopathologic assessment; ypN total, complete number of analyzed lymph nodes; ypN infiltrated, number of affected lymph nodes; ypGrading, tumor grading by histopathologic assessment; R, resectability (0 refers to histologically tumor cell–free surgical margins); cUICC, clinical International Union Against Cancer stage; ypUICC, post-treatment International Union Against Cancer stage; Tis, tumor in situ.

Table 2

List of 54 Most Significantly Changed ($P < .001$) Genes in the T Level Downsizing Class-Comparison Analysis of 23 Patients

Rank	Parametric P	Geometric Mean of Ratios in Responders	Geometric Mean of Ratios in Nonresponders	Fold Difference of Geometric Means	Description	Clone	UG Cluster	Gene Symbol	Map
1	4.40E-06	0.255	0.727	0.351	Calmin (calponin-like, transmembrane)	IncytePD:1 464613	Hs.406099	CLMN	14q32.2
2	4.15E-05	1.37	3.752	0.365	FK506 binding protein 1B, 12.6 kDa	IncytePD:1 288118	Hs.77643	FKBP1B	2p24.1
3	4.88E-05	0.6	1.224	0.49	Kinectin 1 (kinesin receptor)	IncytePD:3 736760		KTN1	
4	5.54E-05	0.853	1.911	0.446	Copine III	IncytePD:3 444952	Hs.14158	CPNE3	8q21.13
5	6.55E-05	9.965	4.943	2.016	LIV-1 protein, estrogen regulated	IncytePD:1 402273	Hs.79136	LIV-1	18q12.1
6	.0001277	0.264	0.832	0.317	Glutamic-pyruvate transaminase (alanine aminotransferase)	IncytePD:1 630709	Hs.103502	GPT	8q24.3
7	.0001571	0.219	0.531	0.412	Incyte EST	IncytePD:1 607471			
8	.0001722	0.657	1.193	0.551	Filamin B, beta (actin binding protein 278)	IncytePD:1 871362	Hs.81008	FLNB	3p14.3
9	.0001874	0.182	0.467	0.39	Villin-like	IncytePD:2 804190	Hs.103665	VILL	3p21.3
10	.0001972	1.654	1.007	1.643	Membrane-bound transcription factor protease, site 2	IncytePD:1 302425	Hs.350970	MBTPS2	Xp22.1-p22.2
11	.000202	0.467	1.256	0.372	Homeobox D9	IncytePD:2 956581	Hs.236646	HOXD9	2q31.1
12	.0002228	0.792	1.565	0.506	CDC42 binding protein kinase alpha (DMPK-like) Homosapiens cDNA	IncytePD:1 602261	Hs.18586	CDC42BPA	1q42.11
13	.000286	0.389	0.698	0.557	FLJ30016 fis, clone 3NB692000429	IncytePD:1 570161	Hs.14931		10
14	.0002972	0.413	0.847	0.488	Monoglyceride lipase	IncytePD:2 174920	Hs.6721	MGLL	3q21.3
15	.0003119	1.357	0.847	1.602	ESTs, glutamate-cysteine ligase, modifier subunit acetyl-coenzyme A	IncytePD:1 432207	Hs.315562	GCLM	1
16	.0003197	0.504	0.805	0.626	Acyltransferase 1 (peroxisomal 3- oxoacyl-coenzyme A thiolase)	IncytePD:1 926543	Hs.166160	ACAA1	3p23-p22
17	.0003223	0.443	0.749	0.591	Regulator of G-protein signalling 19 Interacting protein 1	IncytePD:1 626914	Hs.6454	RGS19IP1	19p13.1
18	.0003379	0.58	1.043	0.556	p21/Cdc42/Rac1-activated kinase 1 (STE20 homolog, yeast)	IncytePD:2 632434	Hs.64056	PAK1	11q13-q14
19	.0003527	0.527	0.829	0.636	Motilin	IncytePD:2 37225	Hs.2813	MLN	6p21.3
20	.0003884	0.594	1.112	0.534	Inositol 1,3,4- triphosphate 5/6 kinase	IncytePD:1 967095	Hs.6453	ITPK1	14q31
21	.0003889	1.728	0.813	2.125	ESTs	IncytePD:4 106720	Hs.12876		6

Rank	Parametric P	Geometric Mean of Ratios in Responders	Geometric Mean of Ratios in Nonresponders	Fold Difference of Geometric Means	Description	Clone	UG Cluster	Gene Symbol	Map
22	.0004007	1.89	1.034	1.828	Hypothetical protein DKFZp762O076	IncytePD:1 967206	Hs.21621	DKFZp762 O076	8q21.3
23	.0004026	0.067	0.155	0.432	Myosin IA	IncytePD:1 502005	Hs.5394	MYO1A	12q13-q15
24	.0004298	0.915	1.876	0.488	S164 protein	IncytePD:2 047730	Hs.180789	S164	14q24.3
25	.0004488	0.968	1.813	0.534	Protein phosphatase 1, regulatory subunit 10	IncytePD:2 314555	Hs.106019	PPP1R10	6p21.3
26	.0004611	1.282	2.452	0.523	KIAA0138 gene product	IncytePD:1 731569	Hs.159384	KIAA0138	19p13.3
27	.0004701	0.396	0.149	2.658	Guanylate cyclase 1, soluble, beta 3	IncytePD:1 417408	Hs.77890	GUCY1B3	4q31.3-q33
28	.0004709	0.242	0.773	0.313	Mucin 5, subtype B, tracheobronchial	IncytePD:1 737280	Hs.102482	MUC5B	11p15
29	.0004967	0.383	0.713	0.537	E74-like factor 1 (ets domain transcription factor)	IncytePD:1 312824	Hs.154365	ELF1	13q13
30	.0005427	0.532	0.826	0.644	Metal-regulatory transcription factor 1	IncytePD:2 467743	Hs.211581	MTF1	1p33
31	.0005701	0.045	0.235	0.191	Small nuclear protein PRAC	IncytePD:2 231168	Hs.116467	PRAC	17q21
32	.0005733	0.619	1.161	0.533	Cyclin T1	IncytePD:2 928577	Hs.279906	CCNT1	12pter-qter
33	.0005792	0.936	1.38	0.678	Hypothetical protein FLJ12949	IncytePD:1 393595	Hs.184519	FLJ12949	19p13.2
34	.0005934	0.489	0.913	0.536	ESTs, moderately similar to TRY2_HUMAN trypsin II precursor (anionic trypsinogen) [H.sapiens]	IncytePD:1 222442	Hs.66915		22
35	.0006109	1.144	2.176	0.526	Homosapiens, clone IMAGE:4797596, mRNA	IncytePD:4 75497	Hs.355279		7
36	.0006261	1.029	0.625	1.646	Eukaryotic translation initiation factor 5A2	IncytePD:7 86494	Hs.104660	EIF5A2	3q26.2
37	.0006281	0.969	0.514	1.885	Interleukin 12A (natural killer cell stimulatory factor 1, cytotoxic lymphocyte maturation factor 1, p35)	IncytePD:2 760318	Hs.673	IL12A	3p12-q13.2
38	.0006662	0.818	1.501	0.545	ESTs	IncytePD:1 398814	Hs.355960		12
39	.0006832	1.043	0.651	1.602	Hypothetical protein MGC26706	IncytePD:2 706048	Hs.65406	MGC26706	Xp22.31
40	.0006906	2.603	1.544	1.686	Incyte EST	IncytePD:2 922438			
41	.0007004	2.038	5.535	0.368	Homosapiens, clone IMAGE:5587702, mRNA	IncytePD:1 964852	Hs.427683		10
42	.0007029	0.899	1.473	0.61	WAS protein family, member 2	IncytePD:1 448116	Hs.288908	WASF2	1p36.11-p34.3
43	.0007109	0.986	2.189	0.45	Adaptor-related protein complex 3, delta 1 subunit	IncytePD:1 301192	Hs.75056	AP3D1	19p13.3

Rank	Parametric P	Geometric Mean of Ratios in Responders	Geometric Mean of Ratios in Nonresponders	Fold Difference of Geometric Means	Description	Clone	UG Cluster	Gene Symbol	Map
44	.0007183	0.101	0.237	0.426	Protein expressed in thyroid	IncytePD:1 807085	Hs.7486	YF13H12	19q13.2
45	.0007203	0.315	1.319	0.239	Sodium channel, nonvoltage-gated 1, beta (Liddle syndrome)	IncytePD:2 121687	Hs.37129	SCNN1B	16p12.2-p12.1
46	.0007569	0.416	0.817	0.509	KIAA0284 protein	IncytePD:1 890138	Hs.182536	KIAA0284	14q32.33
47	.0007649	1.183	0.636	1.86	Solute carrier family 1 (glial high-affinity glutamate transporter), member 3	IncytePD:3 074077	Hs.75379	SLC1A3	5p13
48	.0008029	0.241	0.44	0.548	Deltex homolog 2 (Drosophila)	IncytePD:1 691161	Hs.89135	DTX2	7q11.23
49	.000803	1.236	2.234	0.553	Myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila)	IncytePD:1 692195	Hs.199160	MLL	11q23
50	.0008186	0.29	0.568	0.511	Chromosome 11 open reading frame 13	IncytePD:1 919646	Hs.72925	C11orf13	11p15.5
51	.000825	1.683	2.548	0.661	Small nuclear RNA activating complex, polypeptide 2, 45 kd SMC1 structural	IncytePD:1 445203	Hs.78403	SNAPC2	19p13.3-p13.2
52	.0008488	1.114	1.673	0.666	Maintenance of chromosomes 1-like 1 (yeast)	IncytePD:3 074894	Hs.211602	SMC1L1	Xp11.22-p11.21
53	.0009141	1.708	1.116	1.53	Serine/threonine kinase 18	IncytePD:2 732630	Hs.172052	STK18	4q27-q28
54	.0009645	1.771	1.06	1.671	Platelet-derived growth factor C	IncytePD:9 43826	Hs.43080	PDGFC	4q32

Abbreviation: UG, unigene.

Table 3

Prediction Accuracy Using Microarrays

Array and Patient No.	Response Class Label	No. of Genes in Classifier	Diagonal Linear Discriminant Correct?
cDNA[*]			
1	R	89	No
2	R	48	No
3	R	41	Yes
4	R	45	Yes
5	R	36	Yes
6	R	39	Yes
7	R	40	Yes
8	R	47	Yes
9	R	43	Yes
10	NR	41	Yes
11	NR	39	Yes
12	NR	35	Yes
13	NR	53	Yes
14	NR	38	Yes
15	NR	66	No
16	NR	54	Yes
17	NR	47	Yes
18	NR	40	Yes
19	NR	44	Yes
20	NR	43	Yes
21	NR	84	No
22	NR	46	Yes
23	NR	56	Yes
Oligonucleotide[†]			
24	R	39	Yes
25	R	39	Yes
26	NR	39	Yes
27	R	39	Yes
28	R	39	No
29	NR	39	Yes
30	R	39	Yes

Abbreviations: R, responders; NR, nonresponders.

[*] Percentage correctly classified: 83%.

[†] Percentage correctly classified: 86%.