A Proteomics Analysis of Cell Signaling Alterations in Colorectal Cancer*‡

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To gain further insight into alterations in cellular pathways, tumor profiling, and marker discovery in colorectal cancer (CRC) we used a new antibody microarray specific for cell signaling. Soluble protein extracts were prepared from paired tumor/normal biopsies of 11 patients diagnosed with colorectal carcinoma at different stages; four liver carcinomas were used as a reference. Antibody microarray analysis identified 46 proteins that were differentially expressed between normal colorectal epithelium and adenocarcinoma. These proteins gave a specific signature for CRC, different from other tumors, as well as a panel of novel markers and potential targets for CRC. Twenty-four proteins were validated by using a specific colorectal cancer tissue microarray and immunoblotting analysis. Together with some previously well known deregulated proteins in CRC (β-catenin, c-MYC, or p63), we found new potential markers preferentially expressed in CRC tumors: cytokeratin 13, calcineurin, CHK1, clathrin light chain, MAPK3, phospho-PTK2/focal adhesion kinase (Ser-910), and MDM2. CHK1 antibodies were particularly effective in discriminating between tumoral and normal mucosa in CRC. Moreover, a global picture of alterations in signaling pathways in CRC was observed, including a significant up-regulation of different components of the epidermal growth factor receptor and Wnt/β-catenin pathways and the down-regulation of p14ARF. The experimental approach described here should be applicable to other pathologies and neoplastic processes. Molecular & Cellular Proteomics 6:2150–2164, 2007.

Multiple gene and protein alterations have been associated with every type of cancer. These alterations can be considered as biomarkers and might be useful in detecting cancer, determining prognosis, and monitoring disease progression or therapeutic response. Unfortunately finding new tumoral markers is not an easy task. Only 10 proteins have been described that are effectively used as biomarkers (e.g. prostate-specific antigen, carcinoembryonic antigen, and CA125) (1). Many of them show a lack of sensitivity or present high rates of false positives. New tools and strategies are needed to improve this situation. Protein microarrays in general and antibody arrays in particular offer a combination of high sensitivity and multiplexing possibilities that makes this strategy very attractive for tumor screening and biomarker identification (for a review, see Ref. 2; Ref. 3).

Colorectal cancer (CRC)1 is the most abundant type of neoplasia in developed countries and the second cause of death among cancers. CRC has been well characterized from the genetics point of view (4, 5). It is a relatively slow process, which needs several successive mutations to be present in the tumoral cells and probably takes decades to develop completely. However, this knowledge about the genetic events that are necessary for the progression to carcinoma has not been translated into protein biomarkers. The current Dukes’ staging system for CRC is based on histopathological findings, such as the invasion of the intestinal muscular layer or the adjacent lymph nodes or the metastatic progression. In CRC, most of the tumors detected are already in advanced stages, such as Dukes’ C or D, lowering the estimated survival rate. Genomics studies of CRC involving DNA microarray analysis did not bring new classification tools or improved predictor panels (6–9).

Previously we have carried out studies on differential protein expression analysis based on two-dimensional DIGE gels (10), which enabled us to identify the most abundant proteins in CRC tissues, including some isoforms and post-translational modifications. Although DIGE is very sensitive, low abundance proteins are usually not detectable by mass spectrometry. To overcome this limitation and for a gain in sensitivity, we decided to test an antibody microarray strategy for detecting low abundance proteins. For antibody microarrays and depending on the affinity constant of the antibody, the detection limit can be 2 orders of magnitude below that for DIGE. In addition, the protein/antibody pairs are known “a priori.” Hence microarrays allow for a rapid identification of

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1 The abbreviations used are: CRC, colorectal cancer; TMA, tissue microarray; Ab, antibody; T, tumor; N, normal; FAK, focal adhesion kinase; EGFR, epidermal growth factor receptor; MAPK, mitogen-activated protein kinase; SOTA, Self Organizing Tree Algorithm; pep., peptide; e-NOS, endothelial nitric-oxide synthase; MAP, mitogen-activated protein; JNK, c-Jun NH2-terminal kinase; HRP, horseradish peroxidase; IPA, Ingenuity Pathways Analysis; CLTA, clathrin light polypeptide A; ERK, extracellular signal-regulated kinase.
low expression proteins such as signaling molecules, cell cycle regulators, etc.

The antibody microarray used in these studies contained 224 different antibodies (11), representing markers for eight biological pathways of interest (apoptosis, cell cycle, neurobiology, cytoskeleton, signal transduction, and nuclear proteins). In addition, 12 of the antibodies were specific for particular post-translational modifications (i.e. eight antibodies were phosphospecific for different modifications of death-associated protein kinase, FAK, histone H3, MAPK, p38, PAK1, PYK2, and RAF; and four of them were acetyl-specific for histone H3). Other antibodies were able to discriminate active versus non-active states in functional proteins. These capabilities represent an enormous value in cell signaling characterization, enabling the microarray system to track consecutive nodes of a given cellular pathway, helping to annotate functional variations in those proteins responsible for triggering a cascade of events related to the onset, modification, or conclusion of a cellular process.

In this study, we used an antibody microarray to monitor the changes in the protein expression pattern of tumoral cells from biopsies of patients with colorectal carcinoma as compared with the pattern of normal cells from the surrounding non-affected mucosa. We identified a specific signature for CRC, new markers based on less abundant proteins that might have a potential use in diagnosis, and finally we observed global alterations in the cellular signaling machinery.

**EXPERIMENTAL PROCEDURES**

Samples—Frozen biopsies were collected after tumor resection from 15 patients with CRC, each set of five representing stage A, B, or C according to Dukes’ classification (12). Tumoral tissues and their non-tumoral mucosal counterparts were resected. Samples were collected and made anonymous by the Tumor Bank Network established at the Centro Nacional de Investigaciones Oncológicas (CNIO) according to ethical and legal guidelines. Clinical and pathologic data of the samples are shown in Table I. Reference samples were collected from patients with liver carcinomas.

**Antibody Microarrays—**The Panorama™ Ab Microarray-Cell Signaling was purchased from Sigma-Aldrich. The slide contained 224 antibodies printed in duplicate onto a 32-grid array. Each grid contained seven antibody duplicates plus a Cy3- and Cy5-conjugated BSA positive control and a non-labeled BSA negative control, resulting in 512 spots per slide. In addition, cytoskeleton antibodies to actins, myosins, and tubulins are included for normalization purposes as the expression of these housekeeping proteins does not change with different treatments of sample and control. Alexa Fluor® 555 and 647 fluorescent dyes were from Molecular Probes (Eugene, OR).

**Protein Extraction—**Fifteen pairs of CRC samples and four pairs of liver tumoral samples were initially used for the study. Frozen tissues in optimal cutting temperature were washed twice with chilled PBS and subsequently homogenized by premixing with an UltraTurrax® blender in lysis buffer (0.1% SDS, 10 mm KCl, 1.5 mm MgCl2, and 0.5 mm DTT in 10 mm Tris-HCl, pH 7.5) for 2 min at 4 °C. Proteins were then extracted by three cycles of 30-s sonication in ice. Following that step, interfering components were removed by centrifuging the extracts at 10,000 x g at 4 °C. To assure high purity samples and remove non-protein contaminations, the supernatants were subsequently precipitated and resuspended in 100 µl of 0.1 M sodium bicarbonate, pH 8.3. The labeling reaction occurred by incubation of the reaction mixture with stringing for 1 h at room temperature. The non-conjugated free dye was removed.

### Table I

| Biopsy no. | Tissue | Hospital | Age | Gender | Diagnosis | Color | Location | Dukes |
|------------|--------|----------|-----|--------|-----------|-------|----------|-------|
| 008        | CRC    | H. Ramón y Cajal | 88  | M      | ADC       | S     | LC       | A     |
| 048        | CRC    | H. Ramón y Cajal | 80  | F      | ADC       | S     | LC       | A     |
| 099        | CRC    | H. Ramón y Cajal | 80  | M      | ADC       | C     | RC       | A     |
| 210        | CRC    | H. Ramón y Cajal | 79  | M      | ADC + P   | AC    | RC       | A     |
| 299        | CRC    | H. Ramón y Cajal | 70  | M      | ADC       | AC    | RC       | A     |
| 695        | CRC    | H. Sta. María Rosell | 70 | M      | CA        | S + R | LC       | B     |
| 698        | CRC    | H. Sta. María Rosell | 60 | M      | CA        | R     | LC       | B     |
| 703        | CRC    | H. Sta. María Rosell | 61 | M      | CA        | AC    | RC       | B     |
| 704        | CRC    | H. Sta. María Rosell | 75 | M      | CA        | S + R | LC       | B     |
| 706        | CRC    | H. Sta. María Rosell | 42 | F      | CA        | S     | LC       | B     |
| 711        | CRC    | H. Sta. María Rosell | 81 | M      | CA        | S + R | LC       | C     |
| 713        | CRC    | H. Sta. María Rosell | 82 | M      | CA        | S + R | LC       | C     |
| 715        | CRC    | H. Sta. María Rosell | 72 | M      | CA        | R     | LC       | C     |
| 720        | CRC    | H. Sta. María Rosell | 63 | F      | CA        | R     | LC       | C     |
| 722        | CRC    | H. Sta. María Rosell | 79 | F      | CA        | S     | LC       | C     |

| Gender | Color | Location | Dukes |
|--------|-------|----------|-------|
| M      | S     | LC       | A     |
| F      | S     | LC       | A     |
| M      | C     | RC       | A     |
| M      | AC    | RC       | A     |
| M      | AC    | RC       | A     |
| M      | CA    | S + R    | LC    |
| M      | CA    | S + R    | LC    |
| M      | CA    | S + R    | LC    |
| M      | CA    | R        | LC    |
| M      | CA    | R        | LC    |
| M      | CA    | S        | LC    |

| Age | Gender | Diagnosis | Color | Location | Dukes |
|-----|--------|-----------|-------|----------|-------|
| 88  | M      | ADC       | S     | LC       | A     |
| 80  | F      | ADC       | S     | LC       | A     |
| 80  | M      | ADC       | C     | RC       | A     |
| 79  | M      | ADC + P   | AC    | RC       | A     |
| 70  | M      | ADC       | AC    | RC       | A     |
| 70  | M      | CA        | S + R | LC       | B     |
| 60  | M      | CA        | R     | LC       | B     |
| 61  | M      | CA        | AC    | RC       | B     |
| 75  | M      | CA        | S + R | LC       | B     |
| 42  | F      | CA        | S     | LC       | B     |
| 81  | M      | CA        | S + R | LC       | C     |
| 82  | M      | CA        | S + R | LC       | C     |
| 72  | M      | CA        | R     | LC       | C     |
| 63  | F      | CA        | R     | LC       | C     |
| 79  | F      | CA        | S     | LC       | C     |

**a** Age in years.

**b** M, male; F, female.

**c** ADC, adenocarcinoma; P, Polyp; CA, carcinoma.

**d** C, cecum; AC, ascending colon; S, sigmoid; R, rectum.

**e** LC, left colon; RC, right colon.
from the labeled sample by using Vivaspin concentrators (10,000 molecular weight cutoff, Vivascience), and centrifugation at 7,500 rpm for 5 min. The resulting volumes were adjusted at a final concentration of 1 mg/ml in 100 μl. Dye swapping experiments were performed to evaluate the potential problem of labeling bias with different dyes. Two CRC samples were labeled with alternated dyes, i.e. tumoral and normal samples were labeled alternatively with Alexa Fluor 555 and Alexa Fluor 647, respectively. Correlation coefficient R was determined (R = 0.75).

The extent of labeling was determined by measuring the absorbance of the conjugate solution at 280 and 647 nm (or 555 nm). According to the manufacturer’s instructions optimal performance of the incubation/detection process is achieved at 1 mg/ml labeled sample to get 2–5 mol of dye/mol of protein. To satisfy these ratios 11 CRC and four liver pairs of tumoral samples were finally selected (Supplemental Table S1). Equal amounts of labeled protein of both extracts were incubated on the Panorama Ab Microarray slide for 40 min at a moderate shaking frequency. Then the slide was washed three times in PBS, 0.05% Tween for 5 min and immersed in water for 2 min. Finally the slides were air-dried before scanning with a five-laser ScanArray™ 6000 XL scanner (GSI Lumonics, Ontario, Canada). Images were generated with the ScanArray software.

Bioinformatics Analysis—Microarray images were analyzed with the GenePix™ Pro 4.0 image analysis software. Fluorescence intensity measurements from each array element were compared with local background, and background subtraction was performed. The Alexa Fluor 647/Alexa Fluor 555 ratios were obtained for each experiment, and correlation graphics were plotted to assess the reproducibility of the duplicate spots. Before normalization, spots showing defects were manually flagged. Spots with intensities for both channels (sum of medians) lower than the sum of medians were also discarded. Afterward the Alexa Fluor 647/555 ratio was adjusted to a normalized factor equal to the median ratio value of all spots in the array and then global loess-based normalization was performed using the Diagnosis & Normalization for Microarray Data tool (13). To gain insight into the most significant changes in protein expression, log2 ratios were submitted to preprocessing analysis through PreP (Bioinformatics Unit, CNIO) for filtering, merging of replicates, and imputation of missing values. The ratios of the duplicated spots were averaged and then semilog-transformed. Inconsistent duplicates were excluded. Protein profiles with less than 70% of available data were excluded from further analysis. After applying these criteria the remaining proteins were found suitable for subsequent bioinformatics analysis.

Then to filter flat patterns, protein expression levels were deemed to be up-regulated or down-regulated if the absolute value of the ratio differed by at least 0.7 in 30% of patients. This method selects proteins with large variation in expression levels across the 11 patients and ensures that the proteins considered do show relevant differences with respect to the controls. By requiring that the repression or overexpression be shown by at least 30% of the patients, we make sure that the patterns found are not spurious results from just a few outlying patients. Identification of Alterations in Signaling Molecules—To gain insight into which proteins and sets of proteins distinguish different conditions of tumorigenicity in CRC samples, two levels of analysis were performed: (i) interrogation for differences in protein expression between tumoral and normal mucosa regions from the same patient and (ii) a comparison between CRC versus liver carcinomas to define proteins specifically deregulated in CRC as opposed to other cancer types. In addition, although the number of samples was not high enough for statistical confidence, we compared samples from A, B, and C Dukes’ stages to get some potential clues of molecular differences that support that clinical-pathological classification for CRC cases. Unsualled hierarchical clustering was performed using the Self Organizing Tree Algorithm (SOTA) (14). Proteins were clustered based on normal euclidean distance between them, and conditions in the upper tree were computed according to the UPGMA (unweighted pair group method with arithmetic mean) average algorithm using correlation distance. To visualize protein expression levels the TreeView program was used. Additionally supervised differential expression analysis was achieved using the POMELO II web tool. To identify the proteins that are important for distinguishing CRC from liver tumors or subgroups defined by Dukes’ classification, we carried out t test and analysis of variance test, respectively, with 100,000 random permutations for p value computation. Proteins with unadjusted p values <0.05 were considered the best potential candidates for identifying differential expression among subgroups. In addition to the protein information provided by the microarray manufacturer, other complementary data such as molecular characteristics, sites of expression, interactions, biological functions, implication in diseases, etc. were assigned by using the Human Protein Reference Database (www.hprd.org) (15).

Antibodies—A total of 24 different antibodies were used to validate the microarray results by immunoblotting and immunohistochemistry. The list includes calcineurin, caspase 3 active, β-catenin, caveolin 1, CHK1, clathrin light chain, c-MYC, cytokertin pep. 7, cytokertin pep. 13, desmin, e-NOS, PTK2/FAK phosphorylated Ser-910, GRB2, histone H3 acetylated Lys-9, JNK activated diphosphorylated, MAP kinase ERK1, MDM2, p63, protein kinase B/AKT, prostate apoptosis response 4, S100, and Tau phosphorylated Ser-199/202. Cytokeratin 8.13 and cytokertin 19 were added as controls for CRC. If possible, the same antibodies that were printed onto the microarray according to the manufacturer were used for validation. Source, clonality, and conditions of usage for every case and technique are specified in Supplemental Table S2.

Tissue Microarray Design and Immunohistochemistry—Tissue microarrays (TMA) specific for colorectal cancer with 45 different tumoral samples were prepared as described before (9). Information about clinical and pathological features of the samples used for the TMA can be found in Supplemental Table S3. The arrays were incubated with mono- or polyclonal antibodies against 24 proteins. Specific binding was followed by incubation with anti-mouse or anti-rabbit IgG conjugated with biotin. Visualization of specific interaction was monitored by using the EnVision HRP system (DakoCytomation, Copenhagen, Denmark). For quantification of CHK1 immunostaining, slides were scanned and quantified with the Ariol system (Applied Imaging) by using the Multistain Assy softare. The results are given in percentage of stained nuclei per total number of nuclei in the sample preparation. To assess whether the means of normal group and tumoral group were statistically different from each other; a one-tailed Student’s t test was performed assuming unequal variances. A cutoff of 20 arbitrary units was fixed to remove from the analysis those intensities weaker than background.

Immunoblotting—Protein extracts from normal and tumoral paired tissues from six CRC patients (12 samples in total) were separated in parallel by 10% SDS-PAGE. Proteins were transferred to nitrocellulose membranes (Hybond-C extra) by conventional procedures using semidry equipment (Bio-Rad). After blocking, membranes were incubated with specific mono- or polyclonal antibodies against the 24 selected antibodies listed under “Antibodies.” Membranes were incubated at optimized dilutions (Supplemental Table S2) followed by incubation with either HRP-anti-mouse IgG (Pierce) or HRP-anti-rabbit IgG (Sigma) at 1:20,000 dilution. Specific reactive proteins were visualized with ECL substrate (GE Healthcare).

Signaling Pathway Analysis—Functional pathway and network analyses were generated through the use of Ingenuity Pathways Analysis (IPA) (Ingenuity® Systems). IPA identified the pathways from the IPA library of canonical pathways that were most significant to the data set. Proteins that met the expression ratio cutoff of 1.5 and a p value cutoff

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2 E. R. Morrissey and R. Diaz-Uriarte, personal communication.
of 0.05 for differential expression and were associated with a canonical pathway in the Ingenuity Pathways Knowledge Base were considered for the analysis. The significance of the association between the data set and the canonical pathway was measured in two ways. 1) The ratio of the number of proteins that map to the pathway divided by the total number of proteins that map to the canonical pathway was calculated. 2) Fisher’s exact test was used to calculate a p value determining the probability that the association between the protein in the data set and the canonical pathway could be explained by chance alone.

The network proteins associated with biological functions and/or diseases in the Ingenuity Pathways Knowledge Base were considered for the analysis. The calculation of p scores was used to rank networks on the Ingenuity analysis. The p scores are derived from p values. Say there are n genes in the network, and f of them are Focus Proteins. The p value is the probability of finding f or more Focus Proteins in a set of n proteins randomly selected from the Global Molecular Network. It is calculated using Fisher’s exact test. Because interesting p values are typically quite low (e.g. $10^{-6}$) it is visually easier to concentrate on the exponent. Therefore, the p score is defined as: $p$ score $= -\log_{10}(p \text{ value})$.

RESULTS

Antibody Microarray Performance with Colonic Tissue Extracts—Soluble protein contents were extracted from biopsies from 11 patients diagnosed with colon carcinoma. A mixture containing equal amounts of each labeled extract (5
μg/ml) was incubated on the array. The scanned image for a representative tumoral/normal pair extract is shown in Fig. 1A, which shows the same slide at the two fluorescence emission wavelengths for Alexa Fluor 555 and Alexa Fluor 647. The morphology of the spots, the dynamic range of the intensity response and the signal-to-noise ratio as well as positive and negative controls were routinely checked. These parameters showed consistent correctness (Fig. 1B). The reproducibility of the results was high between duplicate spots on the same slide (Fig. 1C).

**Differential Protein Expression Analysis in Colorectal Cancer with Antibody Microarrays**—We initially searched for proteins with up- or down-regulated expressions with respect to their normal reference mucosa. By using SOTA, a clear distinction between tumoral and normal samples enabled the clustering into two main groups of proteins either up- (in red) or down-regulated (in green). The color code goes from red for overexpressed (+3) to blue (−3) for repressed. White rectangles, data not available. The scale is represented in units.

![Supervised statistical analysis by using POMELO II tool on 11 CRC samples and four cases of liver patient extracts (A) and the same analysis but including the three different Dukes' stages (B).](image)

The color code goes from red for overexpressed (+3) to blue (−3) for repressed. White rectangles, data not available. The scale is represented in units.
## Table II

List of deregulated proteins identified in CRC tissues with antibody microarrays

| No. | Ab commercial name | Protein name | Gene symbol | Entrez gene ID | log2 T/N ratios | T/N -fold change | Diff. expr. p value | Biological function | Validation by IHC/IB |
|-----|--------------------|--------------|-------------|---------------|----------------|-----------------|-------------------|---------------------|---------------------|
| 1   | Apoptosis-inducing factor | Programmed cell death 8 (apoptosis-inducing factor) | PDCD8 | 9131 | -0.958 | 0.515 | 0.0794 | Apoptosis | ND* |
| 2   | ARTS-1 | Type 1 tumor necrosis factor receptor | ARTS-1 | 51752 | -0.688 | 0.621 | 0.4939 | Apoptosis | NDb |
| 3   | Bcl-X | BCL2-like 1 | BCL2L1 | 598 | -0.844 | 0.557 | 0.0308 | Apoptosis | ND |
| 4   | Calcineurin | Protein phosphatase-3, catalytic subunit, α isoform | PPP3CA | 5530 | 0.731 | 1.660 | 0.0249 | Calcium-associated protein | Y |
| 5   | CAM kinase IIa | Calcium/calmodulin-dependent protein kinase IIa | CAMK2A | 815 | -0.698 | 0.616 | 0.1836 | Signal transduction | ND |
| 6   | Caspase 3 | Caspase 3 | CASP3 | 836 | -0.196 | 0.873 | 0.0125 | Apoptosis | ND |
| 7   | Caspase 3 active | Caspase 3 | CASP3 | 836 | 0.762 | 1.695 | 0.0288 | Apoptosis | Y |
| 8   | Caspase 7 | Caspase 7 | CASP7 | 840 | -0.415 | 0.750 | 0.0247 | Apoptosis | ND |
| 9   | Catenin, β 1 | Catenin (cadherin-associated protein), β 1 | CTNNB1 | 1499 | 0.426 | 1.343 | 0.0309 | Cytoskeleton | Y |
| 10  | Caveolin-1 | Caveolin 1 | CAV1 | 857 | 0.611 | 1.527 | 0.2189 | Cytoskeleton | Y |
| 11  | Cdc25 | Cell division cycle 25C protein | CDC25 | 995 | -0.960 | 0.514 | 0.1913 | Cell cycle | ND |
| 12  | Cdc7 kinase | CDC7 cell division cycle 7 | CDC7 | 8317 | -0.894 | 0.538 | 0.7352 | Signal transduction | ND |
| 13  | Cdk6 | Cyclin-dependent kinase 6 | CDK6 | 1021 | -0.920 | 0.529 | 0.0074 | Cell cycle | ND |
| 14  | Clathrin light chain | Clathrin, light polypeptide A | CLTA | 1211 | 0.723 | 1.650 | 0.0065 | Cytoskeleton | Y |
| 15  | c-Myc | Myc proto-oncogene | MYC | 4609 | 0.281 | 1.215 | 0.0127 | Cell cycle | Y |
| 16  | CNPase | 2’,3’-Cyclic nucleotide 3’-phosphodiesterase | CNP | 1267 | -1.010 | 0.496 | 0.2240 | Neurobiology | ND |
| 17  | Cytokeratin pep. 13 | Keratin 13 | KRT13 | 3860 | 0.697 | 1.622 | 0.0832 | Cytoskeleton | Y |
| 18  | Cytokeratin pep. 7 | Keratin 7 | KRT7 | 3855 | 0.537 | 1.451 | 0.0372 | Cytoskeleton | Y |
| 19  | CHK1 | CHK1 checkpoint homolog | CHEK1 | 1111 | 0.342 | 1.268 | 0.0065 | Cytoskeleton | Y |
| 20  | DAXX | Death-associated protein 6 | DAXX | 1616 | -0.214 | 0.862 | 0.0125 | Apoptosis | ND |
| 21  | Desmin | Desmin | DES | 1674 | 0.632/ | 1.550/ | 0.1756 | Cytoskeleton | Y |
| 22  | DOPA decarboxylase | Dopa decarboxylase | DDC | 1644 | -1.154 | 0.449 | 0.4616 | Neurobiology | ND |
| 23  | Dystrophin | Dystrophin | DMD | 1756 | 0.718 | 1.645 | 0.0612 | Cytoskeleton | ND |
| 24  | e-NOS | Endothelial nitric-oxide synthase | NOS3 | 4846 | 0.713 | 1.639 | 0.6441 | Signal transduction | Y |
| 25  | Ezrin | Ezrin | VIL2 | 7430 | -0.902 | 0.536 | 0.4709 | Cytoskeleton | ND |
| 26  | FAK, phospho-pS910 | Focal adhesion kinase 1 | PTK2 | 5747 | 0.861 | 1.816 | 0.4764 | Signal transduction | Y |
| 27  | GRB-2 | Growth factor receptor-bound protein 2 | GRB2 | 2885 | 0.737 | 1.667 | 0.3156 | Signal transduction | Y |
| 28  | Histone H3, acetyl-Lys-9 | Histone 3, H3 | HIST3H3 | 8290 | 0.693 | 1.617 | 0.3268 | Cell cycle/nuclear | Y |
| 29  | Histone H3, phospho-pS10 | Histone 3, H3 | HIST3H3 | 8290 | 0.454 | 1.370 | 0.0378 | Cell cycle/nuclear | ND |
| 30  | JNK | JNK1 protein kinase | MAPK8 | 5599 | 0.336 | 1.262 | 0.0319 | Signal transduction | ND |
| 31  | JNK, activated-pT183, pY185 | JNK1 protein kinase | MAPK8 | 5599 | 0.790 | 1.729 | 0.5504 | Signal transduction | Y |
| 32  | MAP kinase ERK-1 | Extracellular signal-regulated kinase 1 | MAPK3 | 5595 | 0.675 | 1.597 | 0.5218 | Signal transduction | Y |
| 33  | MDM2 | Mouse double minute 2 homolog | MDM2 | 4193 | 0.618 | 1.534 | 0.0678 | Cell cycle | Y |
| 34  | Nedd 8 | Nedd-4-like ubiquitin-protein ligase | WWPT1 | 11059 | 0.635 | 1.553 | 0.0125 | Cell stress | ND |
| 35  | p14 ARF | Cyclin-dependent kinase inhibitor 2A | CDKN2A | 1029 | -1.144 | 0.453 | 0.0795 | Cell cycle | ND |
| 36  | p53 | p53 tumor suppressor | TP53 | 7157 | 0.181/ | 1.134/ | 0.0037 | Cell cycle | ND |
| 37  | p63 | p63 tumor protein | TP73L | 8626 | 0.649 | 1.568 | 0.0382 | Cell cycle | Y |
| 38  | PAR4 | Prostate apoptosis response protein | PAWR | 5074 | 0.583 | 1.498 | 0.0037 | Apoptosis | Y |
| 39  | PKB/AKT | Protein kinase B | AKT1 | 207 | -0.550 | 0.683 | 0.3140 | Signal transduction | Y |
| 40  | Plakoglobin | Plakoglobin | JUP | 3728 | 0.637 | 1.555 | 0.0129 | Cytoskeleton | ND |
regulated (in green) (Fig. 2A). After filtering flat patterns (minimum number of peaks, 3; ratio threshold, 0.7), 18 proteins showed a notable difference in expression between the tumoral and normal colonic mucosa, including 13 overexpressed proteins (Fig. 2B). For many of these proteins, there was a remarkable homogeneity across the 11 CRC samples that was independent of their staging or their localization. Most of the up-regulated proteins presented ratios that ranged between 1.6- and 2.3-fold in almost all the tumoral samples.

When four liver carcinomas were introduced in the analysis, the hierarchical clustering of the 224 antibodies identified several characteristic profiles that differed between the colorectal group and the liver carcinoma group, splitting the samples into three main branches, one for the CRC and the other two for the liver carcinomas (Fig. 2, C and D). This result indicates that different proteins are involved in development and progression of CRC as compared with other cancers, indicating a specific signature for CRC at the protein level. The comparison between CRC and liver carcinomas identified other differentially expressed proteins (p < 0.05); some of them were highly expressed in CRC, such as β-catenin.

Supervised analysis with POMELO II (Fig. 3A) confirmed approximately half of the previously selected proteins while it added other interesting candidates, such as CHK1, caspase 3, or histone H3 phosphorylated at Ser-10 as possible CRC markers. When POMELO II was performed according to Dukes’ staging, the proteins were ranked according to their ability to categorize the samples into the A, B, or C stages (Fig. 3B). Underneath the apparent homogeneity of several proteins along the tumoral samples, POMELO II analysis revealed that all of them showed a differential expression that was statistically significant (p value < 0.05). Moreover there was another set of proteins with less uniform pattern across the distinct stages. The differences for those proteins showed a clear expression trend from early stages A and B to the more advanced C, either revealing protein level decreases (TAU phosphorylated at Ser-199/202, JNK, FAK phosphorylated at Tyr-577, epidermal growth factor receptor, and NFκB) or accumulations in late stages (PAR4, DIABLO, caspase 3, p53, TRF1, and c-MYC).

In total, a list of 46 candidate protein targets was elaborated with the results of the SOTA and POMELO analyses, taking into account variables such as -fold changes, p values, specificity for CRC, and biological function. Among those 46 proteins, 26 were markedly overexpressed in CRC samples, 14 were diminished, and the other six were differentially expressed between stages (Table II).

**Dye Labeling Efficiency**—To address the potential problem of labeling efficiency with different dyes, we carried out a dye swapping experiment. The differential expression analysis made after dye swapping showed a similar pattern in the selected protein targets when compared with the direct labeling method (Supplemental Fig. S1). When clustered by Dukes’ stages, the first five candidates were the same, both in direct labeling and in dye swapping labeling (although some of them had a distinct rank order and slightly different p values). For the 50 first targets, 38 were identical in both lists (76% occurrence). When differential expression analysis was based on tumor type (CRC versus liver), the number of coincidences were 3 of 5 and 40 of 50 (80%), respectively.

**Differentially Up-regulated Proteins Were Verified by a CRC-specific Tissue Microarray Analysis**—We used tissue microarrays to verify the abundance and subcellular localization of those proteins with high expression levels in colorectal cancer. The tissue microarray was assembled with a total of 45 different tumoral samples. We used a panel of 24 antibodies corresponding to the proteins found as overexpressed in CRC in the antibody microarray. Some proteins with a well known association to colorectal cancer (i.e. β-catenin, diphospho-JNK, c-MYC, and cytokeratins 8 and 19) were clearly positive in CRC tumors confirming the microarray data.

At least seven other antibodies showed a clear discriminatory value between tumoral and normal samples. Representative images are shown in Fig. 4. Cytokeratin 13 was positive in 33 of 38 tumors giving a strong cytoplasmic staining. In contrast, cytokeratin 7 was overexpressed in 10% of the tumors. Remarkably there was no overlap between cytokeratin 7 and cytokeratin 13 labeling. Calcineurin gave a strong cytoplasmic staining in tumoral epithelial cells. Clathrin was positive in 29 of 31 (93.5%) tumoral tissues. It gave a granular

| No. | Ab commercial name | Protein name | Gene symbol | Entrez gene ID | log2 T/N ratios | T/N -fold change | Diff. expr. p value | Biological function | Validation by IHC/IB |
|-----|--------------------|--------------|-------------|---------------|----------------|-----------------|--------------------|-------------------|---------------------|
| 41  | Raf1               | Raf proto-oncogene 1 | RAF1 | 5894          | 0.200          | 1.149           | 0.0566            | Signal transduction | ND                  |
| 42  | S-100             | S100 calcium-binding protein, β | S100B | 6285          | 0.465          | 1.380           | 0.0558            | Neurobiology       | Y                   |
| 43  | SMAC/DIABLO       | DIABLO       | DIABLO     | 56616         | 0.796          | 1.736           | 0.0183            | Apoptosis          | ND                  |
| 44  | SMAD4             | MAD homolog 4 | SMAD4 | 4089          | -0.868         | 0.548           | 0.6493            | Cell cycle         | ND                  |
| 45  | Tau_phospo-pS199/202 | Microtubule-associated protein tau | MAPT | 4137          | 0.751          | 1.683           | 0.0072            | Neurobiology       | Y                   |
| 46  | Trf-1             | Telomeric repeat binding factor 1 | TERF1 | 7013          | 0.430/-0.200   | 1.347/-0.871    | 0.0258            | Cell cycle/nuclear | ND                  |

* ND, not done.
* Y, yes.
cytoplasmic staining (from weak to strong) in the tumoral epithelial cells with very little staining of the surrounding stroma and weak reactivity with a few normal tissues (6/38). Anti-phospho-ERK/MAPK3 staining was positive in 20 of 31 cases (64.5%), preferentially in the nuclei with a weaker cytoplasmic staining. Remarkably the staining pattern was polarized and localized in lateral parts of the tumoral areas. A percentage of stromal cells was also stained. There was a weak reactivity with normal tissue that was limited to some nuclei in the basal positions of the crypts. Anti-phospho-FAK/PTK2 (pPTK2)/FAK, and MDM2, using a TMA containing 33 CRC samples. These markers were selected on the basis of a clear discriminatory capacity between tumoral and normal tissues.

**Fig. 4.** Tissue microarray analysis of selected targets in CRC tissues. Shown are images corresponding to the immunohistochemical staining at 200× magnification of seven up-regulated proteins in CRC, cytokeratin 13, calcineurin, CHK1, clathrin light chain, phospho-ERK, phospho-PTK2 (pPTK2)/FAK, and MDM2, using a TMA containing 33 CRC samples. These markers were selected on the basis of a clear discriminatory capacity between tumoral and normal tissues.

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tissues were also stained by this antibody at a lower level but only in the nucleus. For MDM2, 80% of the tumors were positive, showing a nuclear staining in 5–60% of the epithelial tumoral cells. No significant staining was observed in the normal tissues or in the stroma.

Finally CHK1 was positive in 100% of the tumors where 20–90% of the cells showed strong accumulation of CHK1. It gave an intense nuclear and cytoplasmic staining. In the transition areas from normal to tumoral epithelia, staining was more relevant close to the tumor than in luminal apical positions (Fig. 5A). After quantification of the immunostaining, we observed a much higher percentage of stained nuclei in the tumoral preparations (Fig. 5B); assuming an error probability fixed at 5%, we found a t-stat equal to 4.18 and a p value < 0.05, meaning that both populations were significantly different.

Some Up-regulated Proteins Were Not Discriminatory for CRC after TMA Analysis—Some of the deregulated proteins identified by the microarrays failed to provide any discriminatory value for different reasons. Caspase 3 stained mostly necrotic areas. Caveolin staining was positive in the surrounding stroma of the tumor, but it was negative in the epithelial cells. Desmin is a smooth muscle protein that preferentially stained the muscular layers present in the tissues. The inclusion in the tumor samples of parts infiltrating into the muscle might explain its overexpression with respect to normal mucosa. e-NOS was ubiquitously expressed in every tissue, normal and tumoral, and did not allow for a discrimination between them. Histone H3 gave a strong nuclear staining in either normal or tumoral tissues. Thus, its overexpression should be related to the major nuclei density present in tumoral tissues. PAR4 stained nucleus and cytoplasm equally well in tumoral and normal tissues without a clear discrimination. Finally antibodies for proteins S100 and TAU recognized only nervous tissue present in the tumoral samples; this can be considered more an epiphenomenon than a real tumor-associated marker.

Immunoblotting Analysis of Up-regulated Proteins—In those cases where the antibodies were suitable for immunoblotting, we tested their reactivity with CRC samples as a second verification. Protein extracts from normal and tumoral tissues from six patients representing the different stages were resolved by SDS-PAGE and blotted onto nitrocellulose membranes. Unfortunately many of the selected 24 antibodies did not show the required specificity for immunoblotting analysis. Fig. 6 shows the results obtained with CHK1, CLTA, GRB2, KRT7, KRT13, MAPK3, MDM2, PPP3CA (calcineurin),

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**Fig. 5.** Overexpression of CHK1 in CRC. A, immunohistochemical staining of CHK1 in tumor epithelial cells (200× magnification). CHK1 strong overexpression was more relevant close to the tumor than in luminal apical positions. B, CHK1 nuclear staining was quantified in tumoral and normal tissues of the CRC TMA by using the Ariol system (Applied Imaging). Results are given as means ± S.D. of the percentage of stained nuclei in each of the tissue samples. Bars show S.D., p < 0.05.
was observed for p14 ARF, which is a key event in the activation of the EGFR (tyrosine kinase) and the Wnt/β-catenin pathways. highlighted two major pathways, the mitogen-activated protein kinase (MAPK) and the phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathways. The association of the deregulated proteins to cell cycle players was registered (repression of CDC7, CDC25, and CDK6 and overexpression of CHK1) that led us to conclude that normal cell cycle functions were altered in our CRC tumoral samples. Similarly we detected a repression in the levels of caspase 3 and caspase 7, which should be related to a partial inhibition of apoptosis in those tumors. In summary, functional analysis through the use of Ingenuity Pathways Analysis revealed significant changes in cell death, cancer, and cell cycle functions mediated by several of the targets selected in our list of 46 proteins (Supplemental Table S4).

IPA established a multidirectional interaction network between the target proteins, according to biological functions such as cancer processes, cell death events, and connective tissue disorders, as the most significantly related functions. The components of this network are shown in Fig. 8 and Supplemental Table S4.

FIG. 6. Validation analysis of selected CRC targets in tissue samples. A panel of antibodies (Table II) was tested for immunoblotting analysis. Conventional SDS gels were run with protein extracts from normal and tumoral tissues from six patients (two from each Dukes’ stage) using 10% resolving capacity. Proteins were transferred onto nitrocellulose membranes and incubated with specific antibodies against the target proteins. In this figure we collected those antibodies that gave a specific and differential result between normal and tumoral samples.

and PTK2/FAK antibodies. In the case of CLTA, GRB2, MAPK3, and PPP3CA, there was a clear increase in the amount of the up-regulated protein in the carcinoma. Anti-GRB2 serum noticeably recognized the neoplastic component in ⅔ of the paired samples with apparent preference for early stages.

Protein Interactions and Signaling Pathways in CRC Pathogenesis—Microarray data were submitted to IPA for a validation of the obtained results and to visualize complete signaling pathways. The association of the deregulated proteins to canonical pathways highlighted two major pathways, the EGFR (tyrosine kinase) and the Wnt/β-catenin signaling pathways, with a well known association to CRC. Components of the EGFR pathway are well represented in the microarray (Fig. 7A). Many of these components were activated at different ratios; GRB2 activated the Son of Sevenless signaling cascade through c-RAF, MEK1, and ERK1/2 with an increasing up-regulation. This activation will result in cell growth and differentiation. Overall this pathway was the most activated in CRC followed by the β-catenin pathway (Fig. 7B) in which β-catenin and c-MYC are up-regulated. A down-regulation was observed for p14ARF, which is a key event in the activation of tumorigenesis as well as in alterations in cell cycle and apoptosis. In the same way, a consistent picture of perturbations in cell cycle players was registered (repression of CDC7, tubulin, CTNNB1).

DISCUSSION

The use of DNA arrays for profiling mRNA expression levels in biological samples has provided valuable information in many biomedical areas, including different types of tumors. However, in the field of colorectal cancer the results have been below expectations. New approaches are necessary for tumor characterization, classification, and marker discovery. Antibody arrays are now available and can be used for protein expression profiling and biomarker search in tissue samples. Whereas DNA/RNA/oligo arrays give information on the genetic defects that may cause disease, antibody microarrays provide information about protein levels, isoforms, and the corresponding functionality. Although current antibody microarray platforms are limited to a few hundred antibodies, they should improve considerably in the future as more and more specific antibodies are being incorporated in the devices.

Our results showed the existence of a specific signature for CRC at the protein level. Moreover the results indicated a remarkable homogeneity among colorectal neoplasia that was independent of their stage or location. These results would suggest that either more antibodies are required to find specific differences between individual CRC tumors or that sporadic colon cancer is a relatively homogeneous disease that resists further subclassification (16, 17).

Our data provided important clues for the identification of deregulated low abundance proteins and opened new avenues for the study of alterations in cell signaling circuitry in CRC. We found 46 deregulated proteins in CRC of which 25 were clearly overexpressed in tumoral samples versus normal. Some proteins showed a clear discriminatory value by using TMA analysis on different CRC samples representing different stages and locations. These proteins were cytokeratin 13, calcineurin, CHK1, clathrin light chain, MAPK3, phospho-PTK2/FAK (Ser-910), and MDM2. Some of their functional roles and implications in CRC are discussed below.

Little is known about cytokeratin 13 and its presence in...
colorectal cancer. Normal colon epithelium apparently does not express cytokeratin 13, but it has been found in other transitional and keratinized epithelia (18). In contrast, only 5–10% staining was observed for cytokeratin 7 as reported previously for CRC (19). Combinations of cytokeratins 7 and 20 are currently used for defining different subsets of carcinomas (20).

Clathrin-coated structures play a role in the formation of secretory granules and rapid reuptake of membranes after regulated secretion (21). The light chain subunits of clathrin, LCa and LCb, have been implicated in the regulation of coated vesicle disassembly and other aspects of clathrin cycling within the cell. Because the colon adenocarcinomas are secretory tumors, the clathrin activities are probably enhanced, which would explain the abundance of clathrin in the tumoral tissues.

Calcineurin, a calmodulin-dependent serine-threonine-protein phosphatase, is important for Ca$^{2+}$-mediated signal transduction. A recent study described a significant overexpression of calcineurin in colorectal cancer (22), displaying a cytoplasmic staining similar to our observations. These authors suggest that the contribution to malignancy might be mediated through nuclear factor of activated T cells signaling and NFκB activation.

Among the overexpressed proteins, there were several kinases. ERK, a member of the MAP kinase family, is activated by upstream kinases, resulting in its translocation to the nucleus where it phosphorylates nuclear targets. This pathway is constitutively active in several human malignancies and may be involved in the pathogenesis of these tumors (4, 23). In contrast to our observations, some authors (24) reported a repression in ERK1/2 activities in human colorectal cancer. The ERK1/2 MAPK pathway mediates ligand-stimulated signals for the induction of cell proliferation, differentiation, and

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**Fig. 7. Ingenuity pathways analysis.** A, epidermal growth factor (EGF) receptor pathway with indication of proteins whose expression levels appeared up- or down-regulated in our analysis (colored symbols). Gene products in red boxes indicate that the corresponding protein levels are raised in the CRC tumoral biopsies. Gene products in green boxes indicate that the corresponding protein level has been found to be decreased in tumors. Numbers represent log$_2$ T/N ratios of the selected proteins as presented in Table II. Lines indicate protein-protein interactions. Filled arrows and T-bars indicate induction and repression, respectively. Open arrows denote protein translocations, and O-bars designate processes to which the protein at the origin point leads. Vertical ovals signify transmembrane receptors, horizontal ovals indicate transcription regulators, triangles denote kinases, and rhomboids denote enzymes. Small circled “P” and “Ub” represent covalently attached phosphate and ubiquitin groups, respectively. B, β-catenin/Wnt pathway. Symbols are as in A. GAP, GTPase-activating protein; SRF, serum response factor; GBP, guanylate-binding protein; CBP, cAMP-response element-binding protein (CREB)-binding protein; LEF/TCF; lymphoid enhancer-binding factor/T cell-specific factor; APC, adenomatous polyposis coli; SOS, Son of Sevenless.
Cell survival (25, 26). PTK2/FAK gene encodes a tyrosine kinase mainly found in the focal adhesions formed between cells growing in the presence of extracellular matrix constituents (27). Specifically PTK2/FAK regulates cell differentiation, adhesion, migration, and acceleration of the G1 to S phase transition of the cell cycle. FAK becomes heavily phosphorylated on serine residues when cells enter mitosis. Phosphorylations on Ser-843 and Ser-910 are mitosis-specific (28). In fact, mitotic cells were clearly observed by immunohistochemistry with the anti-pFAK antibody in the CRC samples (data not shown). Elevated expression of PTK2/FAK in human tumors has been correlated with increased malignancy and invasiveness (29). Recent findings showed that PTK2/FAK contributes to the secretion of matrix metalloproteinases, representing an important checkpoint in coordinating the dynamic processes of cell motility and extracellular matrix remodeling during tumor cell invasion.

MDM2 is a nuclear phosphoprotein that binds and inhibits transactivation by tumor protein p53 as part of an autoregulatory negative feedback loop (30, 31). The MDM2 protein is a key regulator of cell growth and death and plays a pivotal role in the transformation of normal cells into tumor cells, the hallmark of an oncogene. MDM2 is overexpressed in more than 40 different types of malignancies, including solid tumors, sarcomas, and leukemias (32). Increased MDM2 expression is related to a worse clinical prognosis. MDM2 has been proposed as a diagnostic marker not only for cancer stage but to differentiate between similar cancers (33). In colorectal cancer a high percentage of the tumoral samples were positive for MDM2.

CHK1 protein kinase maintains replication fork stability in cells in response to DNA damage and DNA replication inhibitors. CHK1 is the major cell cycle checkpoint kinase mediating S and G2 arrests in response to various types of DNA damage (34). CHK1 inhibitor has been demonstrated to enhance the cytotoxicity of DNA-damaging agents through abrogation of cell cycle checkpoints (35). A previous study reported CHK1 frameshift mutations and associated truncations in colon and endometrial carcinomas (36). However, there were no previous reports of CHK1 overexpression associated to colorectal cancer. In our study, immunohistochemical analysis gave a particularly strong positive staining in all the tumoral cases. The antibody was not specific for the activated/phosphorylated form of CHK1, recognizing total CHK1. The reasons for this overexpression of CHK1 in CRC are not known but could be due to its role as a guard against genomic instability. CHK1 has been described as essential for maintaining tumor cell viability.

**Fig. 8. Ingenuity pathways analysis of the antibody microarray data.** The most significant connections of a global network consisting of 18 deregulated proteins in CRC (Table II), plus additional interacting molecules, are indicated. Up-regulated proteins are shown in red, and down-regulated proteins are shown in green. The network revealed protein interactions in the context of cell death, cancer, and connective tissue disorders. *EGF*, epidermal growth factor; *Fx*, function; *CP*, cell process.
(37). These results suggest that CHK1 could be an important therapeutic target in CRC.

An interesting aspect of the antibody microarrays is their capacity to provide specific information about the phosphorylation status of the targets. A significant fraction of the highly expressed proteins in CRC were detected as phosphorylated forms (i.e., PTK2/FAK on Ser(P)-910, TAU on Ser(P)-199/202, and PYK2 on Tyr(P)-580, etc.). In fact, some of the active phosphorylated forms of proteins involved in signal transduction displayed the highest scores of expression. FAK phosphorylation on Ser-910 is mitosis-specific and regulated by receptor-mediated pathways (28). FAK and PYK2 are coexpressed in epithelial cells. Phosphorylation of Tyr-579 and Tyr-580 results in maximum PYK2 activation. Both residues are located within the kinase activation loop. A small group of proteins was identified as potential stage-specific markers. For instance, PTK2/FAK phosphorylated on Ser-722 was more abundant in Dukes’ A stages and was decreased in more advanced B and C cases, whereas the same protein phosphorylated on Ser-910 was homogenously overexpressed in all the samples.

Cancer genes and their pathways in colorectal cancer are well characterized (for a review, see Ref. 38). Using the Ingenuity Pathways Analysis we concluded that two pathways, the EGFR and the Wnt/β-catenin, were clearly up-regulated in colorectal cancer tissues. Several potential targets along this pathway might be useful for therapeutic intervention. CHK1 also plays a role in linking this pathway to cell cycle progression. Simultaneously PTK2/FAK mediate the interaction of this signaling pathway with processes such as migration, adhesion, and invasion, which occur at the membrane and extracellular matrix level. The role of calcineurin and clathrin light chain in Ca²⁺-mediated signal transduction, endocytosis, and secretory pathways may also be related to PTK2/FAK interaction. Our study also showed several components of the integrin pathway altered in CRC (caveolin 1, GRB2, MAPK3, PTK2/FAK, PYK2, MAPK8, and JNK).

The family of G protein-coupled receptors that transmit signals through the activation of heterotrimeric GTP-binding proteins constitutes the largest group of cell surface proteins involved in signal transduction. These receptors participate in a broad range of important biological functions and are implicated in a number of diseases. Their signal transduction activity is modulated, among others, by the family of arrestins. Proteins involved in the arrestin β1 pathway, including MAPK3, MDMP, and RAF1 (39, 40), were also altered in our study.

Finally some components of the p53 feedback loops, such as p16INK4a or p14ARF, were down-regulated in tumoral samples. This down-regulation of p14ARF contributes to the observed up-regulation of MDM2 with the subsequent repression of p53 and the increase in tumorigenicity.

In summary, antibody microarrays have shown their usefulness for describing specific protein signatures, to identify new markers using low abundance proteins, and to visualize global alterations of complex signaling pathways. Certainly the power of this technology can be extraordinarily improved as long as new arrays, more focused on specific diseases or carcinomas, are produced and applied in tumor analysis.

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