A gene signature of 8 genes could identify the risk of recurrence and progression in Dukes' B colon cancer patients

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Abstract. The benefit of postoperative adjuvant chemotherapy in patients with Dukes' B colorectal cancer is still uncertain and its routine use is not recommended. The five-year relapse rate is ~25-40% and the identification of patients at high risk of recurrence would represent an important strategy for the use of adjuvant chemotherapy. We retrospectively analyzed gene expression profiles in frozen tumor specimens from patients with Dukes' B colorectal cancer by using high density oligonucleotide microarrays. Our results show a subset of 48 genes differentially expressed with an associated probability <0.001 in the t-test. Another statistical procedure based on the Fisher criterion resulted in 11 genes able to separate both groups. We selected the 8 genes present in both subsets. The differential expression of five genes (CHD2, RPS5, ZNF148, BRI3 and MGC23401) in colon cancer progression was confirmed by real-time PCR in an independent set of patients of Dukes' B and C stages.

Introduction

Colorectal cancer (CRC) is one of the major causes of cancer death worldwide (1). To date, radical surgery followed by adjuvant chemotherapy in patients with node-positive disease (Dukes' C stage), is the mainstay of therapy (2). In the node-negative Dukes' B stage disease no adjuvant chemotherapy is used after tumor resection, although 25-40% of patients will develop recurrent disease; therefore, the role of adjuvant chemotherapy in this setting is still unclear (3). Although the pathological TNM (tumor-node-metastasis) staging system represents the main tool for identifying prognostic differences (4), this system is not sufficient for predicting recurrence in Dukes' B stage (5). There is a need to identify predictive factors, in addition to TNM staging, to guide the identification of Dukes' B patients that are likely to relapse. This would be of great help in improving treatment strategies in the node-negative disease.

In this regard, several molecules have been proposed as predictive markers for colorectal cancer relapse; however, data for most markers remain inconclusive (6). Thus, there are technological limitations for accurately predicting recurrence by traditional methods. High inter-patient heterogeneity of colorectal carcinoma can also limit the predictive ability of tests.

The development of microarray technologies, which allow parallel analyses of many genes, has led to a new era in medical science (7). Several studies have demonstrated that gene expression signatures could predict clinical outcome (8). In colorectal cancer, a study using Affimetrix technology showed a 23-gene-set that represents a prognostic signature inversely associated with a higher risk of tumor recurrence in Dukes' B stage (9). In the present study, we report a gene expression analysis of 16 patients with Dukes' B colon cancer by using spotted microarrays containing 19000 oligonucleotide sequences. Data analysis of microarrays is still a matter of discussion among the scientific community. One of the most worrying issues is the high risk of obtaining false positives. In order to minimize this problem, we used two different statistical procedures in the search of significant differences of gene expression associated with relapse. Initially, we used a permutation t-test for two means and secondly a Fisher test with a variation of the ‘leave one out’ iteration procedure, based on the study presented by Iizuka et al (10). Only the genes selected by both procedures were considered as differentially expressed between relapsed and non-relapsed patients. We obtained a gene-set of 8 genes differentially expressed between both groups.

Validation by real-time PCR was performed for those genes whose probes hybridized with exon regions. We show that down-regulation of BRI3, CHD2, MGC23401, ZNF148 and RPS5 genes is associated with colorectal cancer progression.
Patients and methods

Samples. This study includes 16 samples from Dukes' B colorectal tumors which had undergone surgery at the Department of Surgery of the University Clinic of Navarra, between 1997 and 1999. None of the patients received adjuvant chemotherapy or radiotherapy before surgery or after tumor resection. Six patients developed recurrent disease and the other 10 remained free of cancer 5 years after surgery. For confirmation of the association with recurrence and progression of some genes obtained by gene expression profiling, we analyzed the expression of five genes in an independent prospective set of 27 samples with Dukes' B or C stage by real-time PCR. All tumor samples were collected at resection time and immediately frozen. Staging was performed according to American Joint Committee on Cancer criteria (Greene, FL). The ethics committee of the University Clinic of Navarra approved this study and all patients gave their informed consent.

DNA microarray analysis for gene expression profile. Extraction of total RNA was performed with Trizol® total RNA isolation reagent (Gibco BRL, Life Technologies, Gaitherburg, MD, USA) according to the manufacturer's instructions. All the RNA samples used in this study were cleaned up with RNeasy mini kit (Quigen, Valencia, CA) and were exhaustively treated with RNase-free DNAse I (Quigen) to remove residual DNA. The concentration was quantified using RiboGreen quantification kit (Molecular Probes, Leiden, The Netherlands) and quality control of RNA was performed by electrophoresis and ethidium bromide staining on a 2% agarose gel.

The corresponding cDNA probes were prepared using the Micromax system (NEN, Perkin Elmer, Boston, MA) according to the manufacturer's protocol. The labeled cDNA was pre-heated to 50°C and hybridized to Human 19K Oligo array slides (60 mers) (Center for Applied Genomics, University of Medicine of New Jersey).

After hybridization at 48°C for 16 h in a slide cassette (Telechem, Sunnyvale, CA), slides were washed sequentially in a series of solutions of increasing stringency. Immediately after washing, the presence of fluorescent-labeled cDNAs on the microarray was detected using a fluorescent anti-fluorescein antibody conjugate and TSA detection (Micromax) according to the manufacturer's protocol with appropriate modifications.

Microarray data normalization and analysis. The GMS 418 scanner (Genetic Microsystems, Woburn, MA), a confocal scanning instrument containing 2 laser sources and high-resolution photo multiplier tubes (10 micron resolution) was used for scanning the hybridized microarrays. After image acquisition, the scanned images were imported into ImaGene 4.1 software (BioDiscovery, Marina del Re, CA) to quantify the signal intensities.

The intensity value associated to each spot is the result of subtracting a Gaussian function of the noise from the foreground values (11). After this background subtraction, base 2 logarithms of all data were calculated and genes with more than two missing values were excluded from the analysis. The remaining missing values were replaced by using the KNN imputation method (12). Then, the quantile normalization method (13) was applied to normalize the data.

In silico hybridizations. In order to confirm the identity of the genes able to hybridize with the probes of the genetic signature, we performed two in silico hybridizations with the sequence of the human genome, one with the BLAT algorithm (18) of the University of California Santa Cruz Genome Browser and

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Disease free</th>
<th>Relapsed</th>
<th>P-values</th>
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</thead>
<tbody>
<tr>
<td>Age</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>64.4</td>
<td>62.7</td>
<td>0.77 (ns)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
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<tr>
<td>Female</td>
<td>4</td>
<td>1</td>
<td>0.34 (ns)</td>
</tr>
<tr>
<td>Male</td>
<td>6</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>T stage</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>2</td>
<td>3</td>
<td>3</td>
<td>1 (ns)</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Tumor size</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>&lt;5</td>
<td>7</td>
<td>6</td>
<td>0.37 (ns)</td>
</tr>
<tr>
<td>≥5</td>
<td>3</td>
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</tr>
<tr>
<td>Localization</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>LC</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>RC</td>
<td>3</td>
<td>1</td>
<td>0.81 (ns)</td>
</tr>
<tr>
<td>SC</td>
<td>6</td>
<td>4</td>
<td></td>
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*LC, left colon; RC, right colon; SC, colon-sigma; (ns), not significant.
real-time PCR. RNA extracted from an independent set of 27 patients (15 with Dukes’ B and 12 with Dukes’ C stage) was used for confirmation of the results by real-time PCR. Total RNA (2 μg) from each sample were used to generate cDNA using the Taqman reverse transcription reagent kit (PE Applied Biosystems, Foster City, CA). The reaction mixture was incubated at 42˚C for 30 min, followed by incubation at 72˚C for 10 min.

Each cDNA sample was analyzed in triplicate using the ABI PRISM 7700 sequence detector (PE Applied Biosystems). Real-time PCR was carried out using Taqman Universal PCR master mix (Applied Biosystems), containing ROX to normalize emissions. Primers and probes used for amplification and detection of CHD2, BRI3, MGC23401, ZNF148 and 18S ribosomal RNA were purchased from Applied Biosystems as ‘Assay on demand’ (Assay ID: Hs_00222661_m1 and Assay ID: Hs00854645_g1, Assay ID: Hs00299246_m1, Assay ID: Hs00222661_m1 and Assay ID: Hs_99999907_m1). Primers and Taqman probe for amplification and detection of RPS5 were designed using the Primer Express 1.0 software (Applied Biosystems) (Forward: CTCTAGTGTGGCCACCTCGT, Reverse: CACTGTGTGATGCGGTTCA and Probe: TG CCTTCGAGATCATACACCTGCTCACA). For thermal cycling, the following conditions were applied: 10 min at 95˚C and 40 cycles of 15 sec at 95˚C and 1 min at 59˚C.

Expression levels were normalized to the Ct value of the ribosomal RNA. Fold induction was calculated using the formula 2^-ΔCt, where ΔCt = target gene Ct-‘housekeeping’ gene Ct.

Validation of microarray data by real-time PCR. In order to confirm the role of these genes in CRC progression, we measured the mRNA expression levels of 5 out of 8 genes present in the putative prognostic signature (BRI3, ZNF148, RPS5, MGC23401 and CHD2). RNA from an independent set of patients was tested by real-time PCR. The results of PCR analysis are in agreement with the microarray data (Fig. 3) and confirm that this gene-set signature is associated with colorectal cancer progression.
Figure 2. Unsupervised two-way hierarchical clustering with the 11 gene-set obtained by the variation of the Fisher test for 16 primary tumors of Dukes' B colorectal cancer. Each row is a sample and each column is a gene.

Table II. Signature gene-set differentially expressed between patients with different prognosis.

<table>
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<th>Probe</th>
<th>Gene Function</th>
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<tr>
<td>NM_001271</td>
<td>Chromodomain helicase, DNA binding protein 2 (CHD2) DNA binding, helicase activity, regulation of transcription from Pol II promoter</td>
</tr>
<tr>
<td>NM_001009</td>
<td>Ribosomal protein S5 (RPS5) RNA binding</td>
</tr>
<tr>
<td>AF039019</td>
<td>Zinc finger protein 148 (ZNF148) DNA binding, specific RNA polymerase II transcription factor activity</td>
</tr>
<tr>
<td>NM_015379</td>
<td>Brain protein I3 (BRI3) Involved in TNFα induced cell death</td>
</tr>
<tr>
<td>AF321617</td>
<td>ETS-domain protein ELK4 Transcription cofactor</td>
</tr>
<tr>
<td>AF086427</td>
<td>Unknown Unknown</td>
</tr>
<tr>
<td>AB011118</td>
<td>Hypothetical protein MGC23401 Unknown</td>
</tr>
<tr>
<td>AF085842</td>
<td>ATPase, H+ transporting, lysosomal V0 subunit A isoform 1 (ATP6V0J) Cation transporter activity, hydrogen ion transporter activity</td>
</tr>
</tbody>
</table>

The names of the probes are the GenBank accession numbers of the sequences used to design these probes. Names in bold correspond to down-regulation in relapse, while underline indicates up-regulation in relapse.

Discussion

The gold standard for predicting clinical outcome of most cancers has been clinical and pathological staging of tumors after surgery. However, patients with Dukes' B colorectal cancer at the same tumor stages may show different outcome, indicating that the conventional staging procedures may be unable to precisely predict cancer prognosis. Therefore, the search for new prognostic factors able to identify high-risk patients and modulate cancer treatment options is still actively
ongoing. Although many markers have been extensively described, data for individual molecules failed to elaborate the complex patterns of carcinogenesis and cancer progression. In this regard, the development of microarray technologies, that allow undertaking of parallel analyses of many genes, could help to identify molecular factors involved in cancer progression. In this study, we report an 8-gene signature derived from microarray gene expression data with the aim to identify molecular markers associated with relapse in Dukes’ B colon cancer patients. Our study suggests that colon cancer prognosis can be derived from gene expression profile of the primary tumor. Probably, small changes of different genes in primary tumors are responsible for tumor progression rather than large changes in only one gene.

Probe sequences in silico hybridization with the human genome allowed us to confirm the identity of the genes whose expression was being detected by the probes of our gene signature: CHD2, RPS5, BRI3, ZNF148, MGC23401 (hypothetical protein), ELK4 and GMEB1 or YTFDH2 would be down-regulated in relapse and ATPV0A1 up-regulated. Although most of the probes (5 out of 8) hybridize with exons of the corresponding genes, two of the probes hybridize with introns of two well known genes, ATP6V0A1 and ELK4. Our probe possibly detects a new isoform of these genes in which the introns would become part of exons by alternative splicing. Besides, one probe hybridized only with the intergenic region between the genes glucocorticoid modulatory element B1 (GMEB1) and high glucose regulated protein (HGRP8 or YTHDF2). One of these two genes may present alternative isoforms that include this probe in an exon, or another gene still not described could be between these two genes.

CHD2 is one of the most interesting genes in our gene signature as it has also been related to Dukes’ B colon cancer relapse by an independent group (9) using microarray technology. The CHD gene family is a group of highly conserved proteins sharing sequence motifs and functional domains associated with the regulation of chromatin and gene transcription. It has been recently described that CHD1 specifically interacts with the methylated lysine 4 mark on histone 3 in yeast recruiting histone acetylase activity, what is associated with a remodeling of chromatin that facilitates transcription (20). On the other hand, CHD1 co-immunoprecipitates with the transcriptional corepressor NcoR, with histone deacetylases and also with RNA splicing proteins (21). Thus, CHD1 is a key factor in the regulation of transcription by post-translational modification of histones and also influences alternative splicing. Some authors suggest that the whole chromodomain family may have similar recognition properties; therefore, CHD2 can be expected to participate in the same or similar processes and its down-regulation may affect epigenetics and the whole transcription regulation in the cell.

ZNF148 is a zinc finger transcription factor that is universally expressed (22). It has been shown that ZNF148 binds to GC-rich DNA elements in a variety of promoters involved in growth regulation (23-25). Moreover, for the rat pituitary adenoma cell line GH4, Bai and Merchant (26) showed that elevated expression of ZNF148 inhibits cell proliferation and promotes growth arrest through stabilization of the p53 protein. Furthermore, tumor cells with a mutation in the p53 gene are resistant to ZNF148-mediated stabilization and are associated with hepatocellular carcinoma recurrence (27). In our study, ZNF148 is down-regulated in patients with
relapse and patients with Dukes' C colorectal cancer, suggesting a deficient stabilization of p53 in the nucleus and perhaps a deficient growth arrest and higher rates of cell proliferation.

Ribosomal proteins are highly conserved among eukaryotes and prokaryotes. RPS5 gene encodes a ribosomal protein that is a component of the small 40S subunit of ribosomes. There is recent evidence pointing to extraribosomal functions of ribosomal proteins. In this regard, recent studies have suggested a link between ribosomal biogenesis and cell cycle progression. The molecular mechanism that controls such a link remains obscure. The involvement of ribosomal proteins in stabilizing and activating p53 function has been demonstrated (28). Variable expression of this gene in colorectal cancers compared to adjacent normal tissues has also been observed (29), although so far no correlation between the level of expression and the severity of the disease has been reported. In this study, we found a lower PRS5 expression in patients with relapse in comparison with non-relapsed patients. Moreover, their expression is also down-regulated in Dukes' C patients compared to Dukes' B patients. Additional research will be required to determine the role of ribosomal proteins as checkpoints in the carcinogenesis process in general and of S5 ribosomal protein in colorectal cancer progression.

The BRI3 gene belongs to a family of integral membrane proteins with broad tissue expression. This protein is involved in tumor necrosis factor-alpha-induced cell death in murine fibrosarcoma cells L929 (30). Its overexpression induces apoptosis in these cells and the presence of BRI3 is required in the TNFα-induced cell death. In our study, BRI3 is down-regulated in relapsed patients, suggesting that the TNFα-induced apoptosis pathway could be altered in colorectal cancer progression.

Independently of the confirmation of these hypotheses, our 8 probes for gene expression are able to discriminate between our relapsed and non-relapsed patients. Furthermore, in the present study we showed that the down-regulation of 5 out of 8 genes is associated with colon cancer progression. Thus, a study on the functions of the genes present in this gene signature could also improve our understanding of the molecular mechanisms involved in colon cancer progression.

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References