Original Article
Analysis of differentially expressed genes in colorectal adenocarcinoma with versus without metastasis by three-dimensional oligonucleotide microarray

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Abstract: Background: Our objective was to examine how the gene expression profile of tumor tissue correlates with lymph node metastasis in patients with advanced colorectal adenocarcinoma (CRAC). Methods: We studied 36 patients (20 men and 16 women, 22-90 years of age) treated for CRAC (classifications of T2, T3, or T4; histological grade of G1 or G2). Amplified tumor mRNA samples were exposed to 20,000 human sequence probes and digitized images of the hybridized samples were analyzed. Results: On average, 2389 probes were detected above the background, with an average correlation R value of 0.19 between data from different patient groups (with or without lymph node invasion, colon or rectal, with or without angio-lymphatic invasion, with or without recurrence). Lymph node metastasis had a statistically significant signature according to Significance Analysis of Microarrays (SAM) and parametric t-tests, with a false discovery rate (FDR) = 0.1% and p = 0.001, respectively. Cross-correlation of these two tests identified 102 transcripts as being potentially related to node metastases, with fold changes in the range of 2.182-12.960. Conclusion: We identified 102 differentially expressed genes related to the presence of lymph node metastases in patients with advanced colorectal cancer.

Keywords: Colorectal cancer, gene expression, metastasis, three-dimensional oligonucleotide microarray

Introduction

According to the American Cancer Society, mortality rates for colorectal cancer have decreased over the last two decades, owing to a decrease in the number of cases and improvements in early detection and treatment. When detected early, the 5-year survival rate approaches 90%; however, only 39% of cases are diagnosed early. When there is local or lymph node invasion, the 5-year survival rate falls to 68% [1].

The development of malignant tumors is thought to be the result of sequential changes in various oncogenes and tumor suppressor genes and thus to the proteins they encode [2]. Rarely is a single change in these genes sufficient to cause malignancy. Tumors often have different cytogenetic clones, which originate in cells initially transformed by a genetic change. This heterogeneity contributes to differences in clinical behavior and response to treatment, even in patients with the same histopathological characteristics or diagnosed with the same stage of cancer. Hence, studying carcinogenesis is considered to be of clinical importance to the development of effective treatments for these tumors. Perez et al. [3] noted as early as 1998 the importance of genetics and molecular biology to colorectal cancer. In particular, studies of oncogenes, tumor suppressor genes, and DNA repair genes may yield new perspectives on the diagnosis, treatment, prognosis and follow-up of patients.

Advances in gene expression techniques, such as DNA microarrays have made it possible to quantify genes on a large scale [4-6]. Moreover,
the CodeLink™ platform allows minute differences in gene expression to be detected with 95% confidence [4, 7].

**Background**

The objective of the present study was to examine potential correlations between tumor gene expression and lymph node metastasis in patients with advanced colorectal adenocarcinoma (CRAC). We performed a three-dimensional (3D) analysis of the expression of oligonucleotides (OGNs) whose hyperexpression has been implicated in lymph node metastasis.

**Materials and methods**

**Setting and subjects**

This study was conducted using a database of clinical and histopathological information and biological samples from patients with CRAC, who were treated by the Gastroenterology Surgery Unit of the Federal University of São Paulo (Universidade Federal de São Paulo, UNIFESP) between 2001 and 2008. This database was developed prospectively using the protocol of the inter-institutional Clinical Genome Project for Cancer of the Foundation for Research Assistance of the State of São Paulo (Fundação de Amparo à Pesquisa no Estado de São Paulo, FAPESP) and the Ludwig Institute for Cancer Research. The project was analyzed and approved by the Medical Ethics Committee of UNIFESP-EPM.

This research reviewed and approved by the Ethics in Research Board UNIFESP, on October 26, 2001, with the number CEP 989/01, without restrictions.

Tumor samples for molecular study were collected by a pathologist in the operating room immediately after surgical removal of tumors. After identification, they were immersed in liquid nitrogen and transported for storage at -80°C.

Patients with a T2, T3, or T4 clinical-pathological classification (advanced cancer) were considered for inclusion. The exclusion factors were having received neoadjuvant radio- and chemotherapy. All included patients had low-grade (G1 or G2) tumors. The clinical and histopathological characteristics of the final study sample (N = 36 patients) are detailed in the Results.

**Experimental design**

We compared gene expression between patients with and without lymph node metastases, using the complete sample of 36 patients. We also performed gene expression comparisons between the following subgroups of patients: with versus without angio-lymphatic invasion; localized tumors in the colon versus in the rectum; and with versus without tumor recurrence.

**RNA extraction**

Total RNA was extracted from the tissue specimens with TRizol® (monophasic phenol solution and guanidine thiocyanate; Life Technologies), following the manufacturer’s instructions, purified in silicon columns (Qiagen, Valencia, CA), and evolved in DEPC-treated water. We determined the concentration and purity of each sample was determined by absorption readings at 260 and 280 nm in a Spectronic Genesys 5 spectrophotometer (Spectronic Instruments, Inc., USA). The RNA samples were subjected to electrophoresis in agarose gels with formaldehyde under distilled conditions; the presence of bands corresponding to ribosomal RNA 18 and 28S was considered confirmatory of RNA integrity.

**Gene expression analysis**

Gene expression was analyzed using the CodeLink™ microarray platform with CodeLink™ UniSet Human commercial microarrays (Amersham Biosciences, Piscataway, NJ) containing 20,000 OGNs, with 30 base pairs each, with each of these having a unique access number in GenBank [8]. CodeLink™ software was used to analyze images obtained by a digital scanner; the program attributes a signal-to-noise ratio (SNR) to each probe by calculating a spot average, divided by the background level (median signal produced by empty spaces between spots), at 1.5 times the standard deviation of the background. The spots were labeled as present (flag G) when the SNR was ≥ 1 or as absent (flag L) when the SNR was < 1. In addition, flags are also noted on the basis of other considerations, such as a contaminated spot or contaminated background (C), signal
Expressed genes in colorectal adenocarcinoma by 3-D microarray

saturation (S), irregular form or irregular profile (I), spots removed by the manufacturer (M) or by the user (X). Positive and negative control probes were used to evaluate hybridization efficiency. Along with a set of housekeeping genes, the slides included 68 bacterial probes and 18 positive controls, to allow us to monitor synthesis of cDNA and cRNA, as well as 50 negative controls from the Instituto de Química of USP-SP. Some probes were unique, while others mapped to various genes; this information was important for measuring the reliability of our results and selecting which genes were to be the object of the closest analysis [9].

Reverse transcriptase polymerase chain reaction (RT-PCR) for mRNA amplification

One microgram samples of the total RNA was used to synthesize cDNA. RT-PCR products were purified in the QIAquick® column (Qiagen, Valencia, CA). Complementary RNA (cRNA) was generated by in vitro transcription, using T7 RNA polymerase with Biotin-11-UTP (Perkin Elmer, Boston, MA). The first cDNA strand was generated using SuperScript™ reverse transcriptase, with oligo-dT primers, which have a T7 promoter sequence for RNA polymerase attached at their ends (Figure 1). The second

Figure 1. The 217 genes indicated by t-tests to be differentially expressed in patients with versus and without lymph node metastases at p = 0.001. Degree of gene expression dendrograms produced by the software are shown along the x- and y-axes. Rows correspond to individual genes. Columns correspond to individual patients, with the 9 patients with lymph node metastasis placed together on the right end of the figure. Note the concentration of red color on the right of the graph, showing hyperexpression of genes in patients with lymph node metastasis.
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strand of cDNA was synthesized using DNA polymerase I from Escherichia coli. The cRNA was purified in an RNeasy® column (Qiagen) and quantified by ultraviolet spectrophotometry.

Hybridization
cRNA samples were injected into the microcams of the microarray slides for hybridization, for 18 h at 37°C, with a specific buffer, in an Innova™ 4080 incubator (New Brunswick Scientific, Edison, NJ), at 300 rotations per minute.

Post-hybridization, image capture and analysis

The slides were washed in 0.75× TNT buffer [1× TNT: 0.1 mol/L Tris-HCl (pH 7.6), 0.15 mol/L NaCl, and 0.05% Tween20] at 46°C for 1 h and then incubated with streptavidin-Alexa 647 (Molecular Probes, Eugene, OR) at room temperature for 30 min in the dark. The slides were then washed twice in 1× TNT for 5 min per wash, and then placed in 0.05% Tween20. The slides were dried by centrifugation and stored in the dark. Images were captured and digitized with a GenePix® scanner (Axon, Arlington, TX).

Figure 2. The 119 genes differentially expressed in patients with and without lymph node metastases according to SAM (FDR 0.1%). The layout of this graph follows that of Figure 1.
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and analyzed using CodeLink™ Expression Scanning Software.

Microarray data analysis

The gene expression data were normalized to the intensity median of each slide and the normalized data were exported to Microsoft Excel® spreadsheets. Differential gene expression based on clinical and histopathological characteristics was determined using the parametric t-test [10] and the Significance Analysis of Microarrays (SAM) statistical approach [11].

The data were grouped hierarchically and visualized with SportFire software (TIBCO Inc., Somerville, MA). In the graphs generated, green color denotes less expression of the gene and red color greater expression, with the intensities of these colors being equivalent to levels of expression. We applied the chi-square test to evaluate differences between subgroups. The significance level adopted for our data analysis was p < 0.05.

Results

Patient characteristics

Specimens from 36 patients [16 females (44.4%) and 20 males (55.6%)] with CRAC were included in our analysis. These patients ranged in age from 22 to 90 years old (mean, 61.3 years). Most (33/36) of the patients had T3 class tumors, though 1/36 was classified as T2 and 2/36 were classified as T4.

One-fourth of the patients (9/36) presented with lymph node invasion, and three-fourths (27/26) did not. The number of lymph nodes dissected range from 2 to 24 (mean, 23 nodes). Angio-lymphatic invasion was present in 16/36 (44.4%) of the patients, and absent in 20/36 (55.6%) of the patients. A majority of the patients (21/36) had cancerous lesions only in the colon, while slightly more than a third (14/36) had cancerous lesions only in rectum and a single patient (1/36) had cancerous lesions in both the rectum and the colon. Several patients (5/29; 17.2%) experienced recurrence, though most (20/29; 82.8%) had not. With respect to follow-up, 22/36 (61.1%) of the patients had been followed for at least 12 months since removal of their tumors and 14/36 (38.9%) had been followed for less than 12 months at the time the study was completed. Among those who were followed for more than 12 months, the range of time beyond 12 months was 15-83 months (mean, 41 months). Three (13.6%) of the 22 patients who were followed for at least 12 months died during follow-up.

Differential gene expression

On average, 2,389 probes were detected above the background per sample and, out of the total number of slides analyzed, on average, 12% of the probes had reliable intensities.

As shown in Figure 1, 217 genes were found to be hyperexpressed in patients with neoplastic lymph node infiltration versus those without (p = 0.001, t-test). When we applied the SAM statistical test, we found 119 differentially expressed genes, with false discovery rate (FDR) of 0.1%. The distribution of the intensity of expression of these genes, individually for each patient, can be seen in Figure 2. Patients with lymph node metastasis had hyperexpression of the relevant genes.

As shown in Figure 3, cross-correlation of the two statistical tests (SAM and parametric t-test) identified 102 differentially expressed genes, that is, genes with a statistically significant expression signature (FDR = 0.1% and p = 0.001). Patients with lymph node metastases hyperexpressed these genes (see Supplemental Table 1 for details). An analysis of the global expression of these genes revealed an average fold-change of 5.667 (range, 2.182-12.960). That is, these 102 genes were expressed at a level that was 2.182 to 12.960 times greater than in patients with metastases than in those without lymph node metastases.

In addition, parametric t tests indicated that there were 95 genes that were differentially expressed in tumors from the colon versus tumors from the rectum (p = 0.01). The average fold change between tumors from these two places was 0.66 (range, 0.13-2.31). Parametric t tests also indicated that there were 29 genes that were differentially expressed in relation to the presence or absence of recurrence (p = 0.01). The average fold change between tumors from patients with versus without recurrence was 2.89 (range, 0.24-11.45). Finally, parametric t tests indicated that there were 37 genes
that were differentially expressed between patients with versus without angio-lymphatic invasion (p = 0.01). Thus, 37 genes were differentially expressed between patients with and without angio-lymphatic invasion, with an average difference in expression of 1.056 (range, 0.388-2.112).

Discussion

In the present analysis of gene expression in CRAC tumor samples using the CodeLink™ microarray platform, we identified 102 differentially expressed genes related to the presence of lymph node metastases by cross-correlating the results of two statistical tests, namely SAM and parametric t-tests. Parametric t-tests further pointed to 37 genes that appeared to be differentially expressed with versus without angio-lymphatic invasion, 95 genes that appeared to be differentially expressed in tumors from the colon versus from the rectum, and 29 that appeared to be differentially expressed between patients with versus without recurrence.

Among the transcribed genes with significant differential expression greater than 10 times in

![Figure 3. The 102 genes confirmed by cross-validation of t-tests (p = 0.001) and SAM (FDR 0.1%) to be differentially expressed in patients with versus without lymph node metastases. The layout of this graph follows that of Figure 1.](image)
lymph-node metastasized patient samples versus non-metastasized patient samples (t-test and SAM), we consider the following to be particularly noteworthy: UXT (ubiquitously-expressed transcript); CHCHD2 (coiled-coil-helix-coiled-coil-helix domain containing 2); FAN3D (family with sequence similarity 3, member D); IGJ (immunoglobulin J polypeptide, linker protein for immunoglobulin alpha and mu polypeptides); IFITM3 (interferon Induced transmembrane protein 3); ITN2C (integral membrane protein 2C); MRTS35 (mitochondrial ribosomal protein S35); PRAP1 (proline-rich acidic protein 1) and CLCA1 (chloride channel, calcium activated, family member 1).

**Microarrays and the Codelink™ platform**

Microarray technology is widely used in oncology to elucidate the biological mechanisms of oncogenesis, to discover new medicines, and to develop predictors of outcome, with an aim toward developing individualized treatments for patients [12, 13]. Microarrays are a powerful means of examining an enormous quantity of transcriptions of various genes at the same time. They are electronic systems that analyze DNA fragments and identify the intensity of gene action and metabolic activities. Linking the microarray technique with computer programs and statistical tools has provided important knowledge about areas such as gene expression, pathways mediating cell responses, and tumor classification. Researchers obtain a visual "map" of genetic organization in which hyperexpressed and hypoexpressed genes appear in different colors, while genes that are not differentially expressed appear an intermediate color [14].

Microarrays do have some limitations, such as pitfalls related to image acquisition, variability, classification errors in repeated measurements, and limitations in sensitivity [4]. In addition, it is difficult to compare datasets obtained via different platforms. Studies comparing different platforms have highlighted difficulties in reproducing data both within and across platforms [15-17].

Microarrays employ OGN or DNA probe hybridization to measure the expression of thousands of genes in a single hybridization experiment [11]. Enormous quantities of data are generated, necessitating methods to determine whether observed differences in gene expression are actually significant. Although analysis of microarray data grouping may yield coherent patterns of gene expression, it provides little information about statistical significance. Methods based on conventional tests do not address the probability that a difference in gene expression has occurred by accident. A p = 0.01 criterion for significance, which in the context of experiments evaluating a small number of genes may be acceptable, could lead to the identification of 200 genes by chance in an experiment examining 20,000 genes. This problem led Tusher et al. [11] to develop the SAM statistical method which is specifically adapted for analyzing microarray data.

SAM can identify genes with significant changes in their expression by assimilating a group of specific genes extrapolated by t-tests. Each gene is given a base point for changes in its genetic expression in relation to the standard deviation of repeated measurements for that gene. Genes with scores above a specified threshold are considered to have potentially significant changes in their expression. The percentage of those genes expected to be identified by accident is the FDR. To estimate the FDR, hypothetical genes are identified by an analysis of permutations in their measurements. The limit can be adjusted to identify fewer or greater numbers of genes, and FDRs are calculated for each group. The introduction of SAM has been an important advance given that conventional methods of analysis used FDRs that were between 60 and 80%.

In our study, the estimated FDR for our comparison of samples from patients with versus without lymph node metastases was only 0.1%. In other words, of the 102 genes identified as having altered expression, it is likely that none of them were identified by chance. That is to say, we can have strong confidence that these identified genes have actually undergone biological changes.

For the additional clinical and histopathological data analyzed in this study (location of the tumor, angio-lymphatic invasion, recurrence, and specific mortality), we did not apply the SAM test. These comparisons had p values near 0.01 in parametric t-tests, which we did not consider sufficiently reliable given that when 20,000 genes are being examined, ~200
genes (1% of 20,000) could be tagged as showing significant differences purely by chance. Nevertheless, it is possible that these genes could be important in relation to metastasis of colorectal cancer. Thus, these genes, or subgroups of them, should be evaluated in the future by real-time RT-PCR and immunohistochemistry of new samples.

The guidelines we followed in our study were as follows:

- We used \( p = 0.001 \) rather than 0.01 since the number of genes selected by chance would have been unacceptably high in a platform involving 20,000 genes.

- We observed gene expression in pre-established groups rather than classifying groups according to gene expression.

- We did not form the groups using methods that guaranteed only the best results.

For these reasons, we believe that the results of our microarray study are reliable given the available data. However, we also believe that there is a need for these data to be validated since this is the first study of colorectal cancer, to our knowledge, to use microarrays with the CodeLink™ platform.

In addition to the aforementioned strengths of this work, one additional fact further enhances our findings. Prior studies examining this topic have considered differences in gene expression between groups on the order of 2- or 3-fold to be significant. In our study, significant fold differences were in the range of 2.182-12.960 (mean, 5.667-fold). For example, expression of the LAMC-2 gene was 7.7-fold greater in patients with lymph node metastases than in those without metastases.

Several research groups have identified groups of genes that exhibit a progressive increment of expression favoring changes leading to metastasis. If we start from the premise that microarrays enable us to study thousands of genes and from there develop studies using superarrays (for which few genes have been analyzed, but which are extremely specific with respect to the function and development of carcinogenesis in colorectal cancer), we can confirm that we are on the right path for the development of this line of research.

**Conclusion**

Together with prior works, this study offers a valuable approach for revealing gene expression profiles that allow markers of aggressiveness to be identified. In other types of cancer, such as breast and blood cancers, research programs have reached a more advanced stage; however, in the field of colorectal cancer research, this project is a pioneering one in terms of pointing to the expression of 102 genes that may be involved in carcinogenesis using microarrays, and correlating the findings with histopathological characteristics.

**Acknowledgements**

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**Disclosure of conflict of interest**

None.

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**References**


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Supplemental Table 1. Genes (n = 102) differentially expressed by patients with and without lymph node metastasis.

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<th>Lim U</th>
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<td>0.005</td>
<td>0.086</td>
<td>0.679</td>
<td>2</td>
</tr>
<tr>
<td>26.</td>
<td>1.25/2.44</td>
<td>ID1</td>
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<td>0.086</td>
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**Expressed genes in colorectal adenocarcinoma by 3-D microarray.**