Identification of Novel Markers for the Diagnosis of Malignant Pleural Mesothelioma

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The diagnosis of malignant pleural mesothelioma is difficult, with the most common differential diagnoses being benign pleural diseases and metastatic adenocarcinomas (ADCA). To identify novel markers that would be able to improve diagnostic accuracy, we performed a genome-wide gene expression analysis on tumor cell lines established from pleural effusions (malignant pleural mesothelioma and lung ADCA). This analysis led to the identification of genes encoding novel and pertinent cellular and soluble markers, for which the expression was validated by real-time RT-PCR. Immunohistochemical staining of tumor biopsy specimens with anti–type III collagen antibodies showed positive labeling for mesothelioma cells but not for ADCA cells. Using enzyme-linked immunosorbent assay, we showed that the C-C motif chemokine 2 (CCL2) concentration was significantly higher in pleural effusions from patients with mesothelioma (n = 61) than in subjects with ADCA (n = 25) or with benign pleural effusions (n = 15): median (interquartile range) = 2.99 ng/ml (1.76 to 6.01) vs 0.99 ng/ml (0.51 to 1.83) and 1.47 ng/ml (0.80 to 1.56), respectively, P < 0.0001. Conversely, the galectin-3 concentration was lower in mesothelioma: 11.50 ng/ml (6.73 to 23.53) vs 24.74 ng/ml (20.42 to 70.35) and 17.64 ng/ml (14.81 to 24.68), respectively, P < 0.001. The areas under the curve for CCL2 were 0.8030 and 0.7716 for the differentiation of mesothelioma from ADCA or benign pleural effusions, respectively. Similarly, the areas under the curve obtained for galectin-3 were 0.7980 and 0.6923, respectively. In conclusion, type III collagen, CCL2, and galectin-3 are promising new diagnostic markers for mesothelioma. (Am J Pathol 2011, 178:1033–1042; DOI: 10.1016/j.ajpath.2010.12.014)
other marker or markers may result in a better classification and management of patients.

In recent years, gene expression profiling with large-scale microarrays has been used in many cancers to identify pathways involved in malignant transformation and to find novel candidate diagnostic and prognostic markers. These studies have been conducted with the use of tumor specimens, but this approach requires samples with a high proportion of tumor cells. Furthermore, because biopsy specimens may contain several cell types, expression levels of genes specifically expressed by cancer cells may be hidden or minimized. Microarray results may be biased, which can lead to the identification of markers lacking specificity. To overcome the issue of tumor heterogeneity, some authors have used laser capture microdissection to isolate cell subpopulations. Although it is more specific than microarrays performed with the whole tumor tissue, this technique does not exclude contamination with stromal and immune cells, which generally are in close contact with cancer cells.

The aim of the present study was to perform a genome-wide analysis using a collection of cancer cells isolated from pleural effusions. Our microarray data, confirmed by real-time RT-PCR, led to the identification of several novel genes specifically expressed by MPM cells. Among the newly identified putative markers, we showed that type III collagen may be useful in immunohistochemistry. Furthermore, C-C motif chemokine 2 (CCL2) and galectin-3 levels were measured in an independent set of pleural fluids and proved helpful in differentiating MPM from other pleural diseases.

Materials and Methods

Pleural Fluid Collection

Pleural effusion samples from 101 patients suspected to have or with a recent diagnosis of MPM or metastatic adenocarcinoma (ADCA) were collected aseptically at the Laënnec Hospital (St-Herblain, France) between 1998 and 2010. Samples were centrifuged at 2500 rpm in a Heraeus Multifuge for 20 minutes at +4°C, and supernatants were aliquoted and stored at −80°C. All patients gave informed consent and had not received any prior chemotherapy. Pathologic diagnosis relied on both fluid cytology and immunohistochemical analysis of pleural biopsies obtained by thoracoscopy. Final diagnosis, established by the anatomopathologic department (Laënnec Hospital, St-Herblain, France) and confirmed by the Pathology French Group for Assistance in the Diagnosis of Mesothelioma, divided the patients into three groups (Table 1): 61 patients with MPM, 25 patients with pleural metastasis of various carcinomas (ADCA group), and 15 patients with benign pleural effusions (BPEs).

Cell Lines

Mesothelioma and lung ADCA cell lines were established from pleural effusions collected by thoracoscopy with cancer. Briefly, cell pellets obtained after centrifugation of pleural fluids were resuspended in sterile Roswell Park Memorial Institute-1640 medium (Sigma-Aldrich, Saint-Quentin Fallavier, France). Erythrocytes were removed by density gradient centrifugation in Ficoll-containing media lymphocyte separation medium 1077 (PAA Laboratories, Les Mureaux, France). Cells were washed twice in Roswell Park Memorial Institute-1640 medium and plated in six-well plates at a density of 2.10^6 cells/well. Cells were cultured in Roswell Park Memorial Institute-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mmol/L L-glutamine, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin (all reagents from Sigma-Aldrich). Two days after plating, nonadherent cells were removed by thorough washing. Tumor cells grew as monolayers and could be maintained in culture for up to 30 passages without any obvious change in morphology, phenotype, and behavior. Tumor cells were characterized for the expression of a panel of markers commonly used for the differential diagnosis of MPM (see Supplemental Figure S1 at http://ajp.amjpathol.org). The small-cell lung carcinoma (H69) and lung carcinoma (A549) cell lines were purchased from ATCC (LGC Promochem, Molsheim, France).

Immunofluorescence

Tumor cells were cultured on glass coverslips until 50% to 60% confluence was achieved. Cells were fixed with

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>MPM (n = 61)</th>
<th>ADCA (n = 25)</th>
<th>BPE (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, year (mean ± SD)</td>
<td>67.8 ± 10.2</td>
<td>66.4 ± 13.4</td>
<td>70.1 ± 12.6</td>
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<tr>
<td>Male sex, n (%)</td>
<td>51 (83.6)</td>
<td>7 (28)</td>
<td>13 (86.6)</td>
</tr>
<tr>
<td>Confirmed asbestos exposure, n (%)</td>
<td>27 (44.2)</td>
<td>3 (12)</td>
<td>4 (26.6)</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epithelioid</td>
<td>49</td>
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</tr>
<tr>
<td>Sarcomatoid</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mixed type</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unspecified</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary tumor, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung ADCA</td>
<td>10 (40)</td>
<td></td>
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<tr>
<td>Breast ADCA</td>
<td>6 (24)</td>
<td></td>
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<tr>
<td>Ovarian ADCA</td>
<td>3 (12)</td>
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<tr>
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<tr>
<td>Unknown origin</td>
<td>4 (16)</td>
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RNA Isolation and Labeling

Total RNA was isolated and treated with deoxyribonuclease I using the Ribonuclease-Free Deoxyribonuclease Set and the RNeasy Mini Kit according to the manufacturer’s protocol (Qiagen, Courtaboeuf, France). RNA integrity was assessed using an Agilent 2100 bioanalyzer (Agilent Technologies, Massy, France). The Amino Alkyll MessageAmp II aRNA Amplification Kit (Ambion, Courtaboeuf, France) was used to generate amino allyl RNA, which was subsequently labeled using the Cy3 Post-Labeling Reactive Dye Pack (Amersham Biosciences, Piscataway, NJ). A reference sample, which consisted of a pool of equal quantities of amino allyl RNA from all biological samples, was Cy5-labeled.

Microarrays

Microarrays covering the entire genome were prepared in-house using 50-mer oligonucleotide probes obtained from the Ocimum human genome–wide microarray collection (Set A) (Ocimum Biosolutions B.V., IJSSELSTEIN, THE NETHERLANDS). Probes were diluted to a final concentration of 20 μmol/L in printing buffer (Buffer A from Ocimum Biosolutions) and printed onto epoxy-silane-coated glass slides (slide E–Schott-Nexterion; Schott, Clichy, France) using a Lucidea Arrayer (GE Health Care, Orsay, France) for 15 minutes at 37°C for 30 minutes. Subsequently, 40 μl of each sample were pipetted onto the chips. Hybridization was performed at 42°C for 17 hours using a Glass Array Hybridization Cassette (Telechem, Sunnyvale, CA). Finally, the slides were washed in the following washing solutions (2× SSC, 0.1% SDS; 1× SSC; 0.2× SSC; 0.05× SSC) and dried by centrifugation. Microarray data are available in the Gene Expression Omnibus archive (accession GSE17310).

Data Analysis and Statistics

Hybridization and background signals, as well as quality control parameters, were measured with use of GenePix Pro 5.0 image analysis software (Molecular Devices, St-Gregoire, France). Technical biases were corrected by a Lowess normalization with use of a channel-by-channel procedure.17,18 For each microarray, Cy3 and Cy5 signal intensities were individually normalized to the median profile of all Cy3 or Cy5 signal intensities (values below background were filtered out). The expression level of each gene was calculated as log2(Cy3/Cy5) so that relative variations, rather than absolute values, were used for interpretation. Gene expression profiles were used to classify genes and biological samples using a hierarchical clustering method with the Gene Cluster program (average linkage clustering using Pearson correlation coefficient as similarity metric).19 Results were displayed using the Java TreeView program (version 1.1.3 for Windows available at http://jtreeview.sourceforge.net).20 Validation of the most significant differences was carried out using a Student’s t-test for unpaired samples. Multiple testing corrections were applied to reduce the number of false-positive results using the Benjamini and Hochberg False Discovery Rate. Genes were classified according to their adjusted P value and their difference in relative expression (fold-change MPM vs ADCA).

Real-time RT-PCR

One microgram of total RNA was reverse-transcribed with use of Moloney-Murine Leukemia Virus Reverse Transcriptase (Invitrogen). PCR reactions were performed with use of QuantiTect Primers (Qiagen) and RT2 Real-Time SYBR-Green/ROX PCR Mastermix (tebu-bio, Le Perray-en-Yvelines, France), according to the manufacturer’s instructions.

Immunohistochemistry

Immunohistochemistry was performed on paraffin-embedded tumor tissue samples with use of standard techniques. Primary antibody anti–type III collagen (Sigma-Aldrich) was used at a dilution of 1:100. A
positive result was defined as the presence of cytoplasmic staining in tumor cells.

**Enzyme-Linked Immunosorbent Assays**

Pleural levels of MCP-1 and galectin-3 were determined using commercial enzyme-linked immunosorbent assay kits (Promokine, Heidelberg, Germany), according to the manufacturers' instructions, and results were expressed in ng/ml. Pleural samples were assayed in duplicate at a dilution of 1:10.

**Statistical Analysis**

All analyses, except for those from the microarray experiment, were performed with use of the Graphpad Prism software (Graphpad Software, La Jolla, CA). Data from the real-time RT-PCR experiments were compared with use of the non-parametric Mann-Whitney test. Data from enzyme-linked immunosorbent assays are reported as median and interquartile range. Comparisons between groups were performed with use of the Kruskal-Wallis test followed by Dunn's post test. Receiver-operator characteristic (ROC) curves were used to quantify marker performance and to provide estimates of sensitivity for a given specificity. Areas under ROC curves (AUC), reported with their 95% confidence intervals, were calculated as an overall performance indicator of the biomarkers. The best statistical “cut-offs” were calculated by minimizing the distance between the point with specificity = 1 and sensitivity = 1 and the points on the ROC curves.

**Figure 1.** Hierarchical clustering of data from the microarray analysis of gene expression in tumor cell lines from patients with MPM and ADCA. The dendrogram representing the arrangement of samples is shown on the right. Individual gene expression levels (in rows) for each sample (in columns) were normalized and expressed relative to the median value for each gene in all samples. Genes expression ratios are represented as follows: red (high) > black > green (low).
Results

Identification of Genes Differentially Expressed Between MPM and ADCA

For the microarray experiment, we included a total of 51 samples, corresponding to biological replicates of 17 cancer cell lines. Because cell phenotype might evolve along with cell subculture, each cell line was included in the study at three different passages after thawing of the initial vials. Cells were used between the fifth and 25th passage. We examined the global gene expression profiles of these cell lines by performing a two-dimensional, unsupervised cluster analysis (Figure 1). The dendrogram, specifying the arrangement of samples, revealed that all MPM and lung ADCA samples grouped together. However, three MPM specimens (meso122, 34, 52) were on the same branch of the dendrogram as the ADCA specimens, highlighting the difficulty of distinguishing these pathologies, even with the use of microarrays.

To identify markers that could best distinguish lung ADCA from MPM, we kept genes represented on the microarrays with a high significant difference (adjusted $P < 0.01$; fold-change $\geq 2$) with regard to the average expression levels between both tumor cell types. Under these conditions, we found 74 genes specifically overexpressed in MPM cell lines, whereas only nine were overexpressed in ADCA cell lines (Figure 2). A list of selected genes differentially expressed between ADCA and MPM cell lines, ranked according to their fold-change, is shown in Table 2. Among the genes overexpressed in mesothelioma cells, COL3A1, SLPI, and ITLN1 displayed the highest fold-change. CDH11, MYL9, ANXA8, COL3A1, C4orf49, BDKRB1, UPK1B and CD200 displayed very low $P$ values ($\leq 10^{-7}$), indicating that there might be only a weak overlap between MPM and ADCA samples for these genes. On the contrary, the genes that were most significantly overexpressed in ADCA cells, such as LGALS3 or OCIAD2, displayed relatively higher $P$ values.

Validation of Microarray Data

We used real-time RT-PCR to confirm the expression levels of selected genes by comparing cDNA generated from total RNA from each cell line used in the microarray (pool of each triplicate), as well as cDNA from two other lung cancer cell lines (A549 and H69) (Figure 3). As expected, we found that RT-PCR results reflected those obtained with microarrays, but most of the time with a higher fold-change, especially when genes were barely detectable (ct $> 35$). We confirmed that COL3A1, SLPI, ITLN1, and several other genes shown in Table 2 are overexpressed in MPM cells (compared with lung cancer cells), while OCIAD2, UPP1, and LGALS3 transcripts are more abundant in lung cancer cells.

Validation of COL3A1 as a Suitable Marker for Immunohistochemistry

Our microarray experiment led to the identification of COL3A1 as being overexpressed in mesothelioma cells compared with lung ADCA cells, with the highest fold-change and a low $P$ value ($5.97 \times 10^{-7}$). This gene encodes pro-alpha1 chains of type III collagen, which
is a fibrillar collagen usually found in extensible connective tissues. Immunohistochemistry for COL3A1, performed on an independent panel of 11 tumor biopsies (5 MPM and 6 ADCA), showed a granular, cytoplasmic staining in 100% of mesothelioma cells (Figure 4). This staining was noted as weak/moderate (+) and moderate/strong (++) in 60% and 40% of mesothelioma cells, respectively. Conversely, no staining of ADCA cells was observed in the lung tumors examined (only the stromal compartment was immunostained).

**Assessment of the Usefulness of CCL2 and Galectin-3 in Pleural Fluids**

Of the genes identified in this study, several encode soluble markers that may be released into the pleural fluids. We chose to analyze the levels of C-C motif chemokine 2 (CCL2) and galectin-3. CCL2 is a chemokine with potent immunoinhibitory effects that recently has been proposed as a valuable target for immunotherapy.\(^{21,22}\) Galectin-3, encoded by LGALS3, is a multifunctional protein that has been implicated in tumor invasion and metastasis.\(^{23}\) The levels of these two proteins were assessed in an independent set of pleural fluids (Table 1). We found that the CCL2 concentration was significantly higher in patients with mesothelioma (n = 61) than in subjects with ADCA (n = 25) or BPEs (n = 15) (Figure 5A): median (interquartile range) = 2.99 ng/ml (1.76 to 6.01) vs 0.99 ng/ml (0.51 to 1.83), respectively, P < 0.0001. Conversely, the galectin-3 concentration was lower in mesothelioma than in ADCAs: 11.50 ng/ml (6.73 to 23.53) vs 24.74 ng/ml (20.42 to 70.35), P < 0.0001 (Figure 5B). However, galectin-3 may not be useful to distinguish MPM from BPE (17.64 ng/ml (14.81 to 24.68)). ROC analysis of CCL2 and galectin-3 showed an AUC of 0.8030 (95% confidence interval: 0.7061 to 0.9003) vs 0.7980 (0.7019 to 0.8942) for differentiating MPM and ADCA (Figure 5C), with best cut-offs of 1.79 ng/ml (sensitivity = 78.0%, specificity = 19.97 ng/ml (sensitivity = 80.0%, specificity = 73.8%), respectively. The AUC of CCL2 for MPM vs BPE was 0.7716 (0.6429 to 0.9003) for CCL2 (Figure 5D).

**Discussion**

Despite recent improvements, the diagnosis and treatment of MPM is still a major challenge. Because of the unspecific symptoms and tissue polymorphism of this cancer, MPM can be confused with benign pleural dis-
Novel Markers for Mesothelioma Diagnosis

Figure 3. Validation of microarray data. Real-time RT-PCR was performed for selected genes in mesothelioma (MPM) and lung cancer (LC) cells. Individual mRNA levels were normalized to RPLP0 (ribosomal phosphoprotein P0 housekeeping gene), and statistical analyses were performed using the Mann-Whitney test (black bars represent the median values). *P < 0.05; **P < 0.01; ***P < 0.001.

- Lung adenocarcinoma
- Lung carcinoma (A549)
- Small-cell lung carcinoma (H69)
- Epithelioid mesothelioma
- Sarcomatoid mesothelioma

eases or with pleural metastasis of ADCAs. Indeed, the performance of diagnostic markers in identifying mesothelioma cancer cells is still questionable. Various immunohistochemical markers are currently available. However, their value in the correct identification of MPM cases using biopsy samples (or cells present in pleural effusions) is, to some extent, limited by nonspecific staining, focal staining, or poor staining quality. More re-
Recently, promising soluble markers, such as mesothelin and osteopontin, have been proposed, but their clinical utilities are still under investigation.24 Because an accurate diagnosis is crucial, both for the management of patients with MPM and also for the settlement of financial compensation for individuals with a history of asbestos exposure, the development of new tools and the improvement of existing diagnostic procedures are of major interest.

Because the presence of a pleural (unilateral) effusion indicates MPM in the majority of patients, we evaluated the possibility of using malignant cells from pleural fluids in a genome-wide analysis with the goal of identifying novel and specific markers to facilitate an easier identification of MPM. Most of the microarray studies reported to date have been performed using tumor biopsy specimens, but such profiling can be compromised by contamination with different cell types. Furthermore, some authors have reported difficulties in performing gene expression studies on cells contained in pleural fluids, essentially because of RNA “weakness” of their samples.25 In our study, we used tumor cell lines isolated from pleural effusions of patients with MPM or ADCA and characterized for the expression of a panel of diagnostic markers. Gene expression profiling of a panel of 17 cell lines revealed a good segregation between samples, in agreement with the initial classification made by immunohistochemical staining of corresponding pleural biopsy specimens. Based on both statistical and biological criteria, we identified 83 genes differentially expressed between MPM and lung ADCA. It is interesting that calretinin (encoded by CALB2) and podoplanin (encoded by PDPN), two validated immunohistochemical markers for MPM diagnosis, also were identified in our study as being overexpressed in mesothelioma compared with lung ADCA cells.

The clinical signs of MPM are usually nonspecific and should not be used alone as diagnostic criteria, even in the case of previous asbestos exposure. It is recommended that the diagnosis of MPM should always be based on immunohistochemical evaluation.27 To discriminate MPM from ADCA, it is recommended that two markers with positive diagnostic value and two markers with negative diagnostic value be used. In the present study, COL3A1 was shown to be a novel, interesting positive marker for MPM, potentially useful in immunohistochemistry. Indeed, we showed that anti-COL3A1 antibody stained mesothelioma but not lung ADCA cells in the 11 tumors analyzed. Of interest, in one case of mixed subtype mesothelioma, both epithelial and sarcomatoid cells were found to be positive for COL3A1. These results are encouraging and should be confirmed in a larger number of samples, including other tumor types and atypical mesothelial hyperplasia.

During the past few years, several attempts have been made to identify soluble molecules that can aid in the

![Figure 4](image4.png)

**Figure 4.** Immunohistochemical staining of tumor tissues from four patients with mesothelioma (magnification: ×200 (A and B) or lung ADCA (magnification: ×200 (C); ×400 (D)), using antibodies specific for COL3A1. Cyttoplasmic staining can be detected in mesothelioma cells but not in lung ADCA cells. Note also the positive staining of the stroma (C).

![Figure 5](image5.png)

**Figure 5.** CCL2 and galectin-3 levels in pleural effusions from patients with mesothelioma (MPM), metastatic adenocarcinoma (ADCA), or in benign pleural effusions (BPE) (A and B). Horizontal bars are median values. Dashed lines represent proposed cut-off values. Statistical significance was calculated using the Kruskal-Wallis test followed by Dunn’s post-test (**P < 0.01, ***P < 0.001). ROC curves for CCL2 and galectin-3 in differentiating MPM and ADCA (C) or MPM and BPE (D).
early diagnosis of MPM with the use of serum and pleural effusion samples. Recent reports have shed light on the utility of two possible markers, soluble mesothelin (or SMRP) and osteopontin.

Mesothelin is expressed on the surface of normal mesothelial cells and is overexpressed in various cancers, including MPM, ovarian carcinoma, and lung carcinoma.

Our group has recently shown that the release of soluble mesothelin (SMRP) into the pleural fluids and blood of patients may result from both aberrant splicing and protease cleavage by matrix metalloproteinases and/or a disintegrin and metalloproteinases. However, SMRP levels are low in non-epithelioid MPM, and its poor sensitivity limits the value of SMRP as a unique marker.

Combining SMRP with other potential markers has been suggested, but no combination with other known markers (eg, hyaluronic acid, CA125, and osteopontin) has been proven useful for improving SMRP value in MPM diagnosis. In the present work we also focused our attention on genes encoding soluble/secreted proteins, the expression of which could help to distinguish MPM from other pleural diseases. Our attention was retained by CCL2, also known as monocyte chemoattractant protein 1, because this chemokine with pro-tumorigenic and immunomodulatory effects could also be used as a therapeutic target.

In fact, it was shown recently that overexpression of CCL2 by tumor cells injected into the pleural cavity of immunocompetent mice led to increased malignant pleural effusion volumes and reduced survival. Furthermore, CCL2 blockade reduced tumor growth and increased CD8 T-cell response in mouse models.

Here we have shown that CCL2 levels are significantly higher in pleural effusions of patients with MPM (including sarcomatoid and mixed subtypes) than in patients with metastatic cancer (not only of lung origin) and patients with benign pleural effusions. The ROC curve analysis of CCL2 for differentiating MPM from ADCA indicated an AUC of 0.8030, a slightly higher than in patients with metastatic cancer (not including patients from another hospital). We found that galectin-3 levels in patients with lung cancer were even higher than in the other cancers: median (interquartile range) = 37.17 ng/ml (22.08 to 79.47). The AUC was also higher (0.8721, 95% confidence interval = 0.7961 to 0.9442), indicating that galectin-3 may be a very good marker for lung ADCA. In this case, the best cut-off value (15.24 ng/ml) gives 100% sensitivity and 67.21% specificity.

In conclusion, this study suggests novel, putative markers that may facilitate the diagnosis of MPM. Although all potential candidate genes have not yet been tested, we have shown that type III collagen, CCL2, and galectin-3 may be useful, either for immunohistochemical analyses of tumor tissues or for the assessment of soluble factors in pleural effusions. The data presented here are promising but warrant further multicenter investigations with larger series of patients.

In addition, the diagnostic and prognostic value of serum (or urinary) CCL2 and galectin-3 levels should be evaluated.

Acknowledgments

We thank Dr. Remi Houlgatte and Catherine Chevalier (Plateforme Puces à ADN de Nantes) for their help and expertise in setting up the microarray experiment and Raluca Teusan, Audrey Bihoué, and Edouard Hirchaud for bioinformatic interpretation and analysis. We also thank Dr. Jean-François Fonteneau for his critical reading of this manuscript.

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