Quantitative Analysis of Aldolase A mRNA in Liver Discriminates between Hepatocellular Carcinoma and Cirrhosis

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Background: Chronic liver diseases can progress to cirrhosis and to hepatocellular carcinoma. Timely and unequivocal recognition of the neoplastic evolution of cirrhosis is critical. To this aim, we used a noncompetitive reverse transcription-PCR procedure to analyze aldolase A mRNA in liver tissue from patients with chronic liver diseases at different stages.

Methods: We studied 12 patients with hepatocellular carcinoma, 19 patients affected by chronic hepatitis C or cirrhosis, and 7 healthy controls. Aldolase A mRNA was reverse-transcribed to cDNA, which was then amplified by PCR. The amplified segments were “read” with a novel dot-blot procedure. A calibrator with the same sequence, synthesized in vitro using a T7 phage promoter, was processed at scalar dilutions in parallel to the target samples to generate a calibration curve and so quantify the target mRNA (detection limit, 0.03 amol; linearity spanning five orders of magnitude).

Results: Aldolase A mRNA was ~10-fold higher in liver biopsies from patients with hepatocellular carcinoma vs patients with chronic hepatitis C or cirrhosis, and healthy individuals. Furthermore, aldolase A mRNA concentrations were 1.2- to 21.3-fold higher in 12 liver biopsies compared with the paired surrounding cirrhotic tissue.

Conclusions: The quantitative analysis of liver tissue aldolase A mRNA differentiates between nonneoplastic chronic liver diseases and hepatocellular carcinoma, which suggests that it has diagnostic potential.

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Chronic liver diseases are evolving entities that give rise, in a percentage of cases, to hepatocellular carcinoma (HCC); obviously, the earlier the cirrhosis/HCC switch is identified, the less dismal the clinical outcome. Despite their sensitivity, imaging techniques give clear positive results in ~65% of HCC lesions (1), and the subsequent cytological procedures can fail to distinguish cirrhotic from HCC lesions (2). Although the neoplastic evolution of cirrhosis is associated with various biochemical alterations (3), including the resurgence of several genes that are expressed only in fetal tissue, e.g., α-fetoprotein (4) and aldolase A (5), serum protein analysis scarcely contributes to the identification of neoplastic transformation of cirrhosis.

Therefore, because quantitative analysis of specific mRNAs is the most efficient procedure for evaluating the extent of gene expression, we used this approach in a variety of clinical situations to identify metastatic cells expressing aberrant messenger species in blood (6), and to monitor minimal residual disease (7) and the effect of therapy in patients with lymphoproliferative disorders (8). The analysis of specific mRNA has also been used to identify metastatic cells in bone marrow (9) and to analyze oncogene expression in human cancer (10).

Various procedures have been devised to quantitatively analyze specific mRNAs based on the reverse transcription of mRNA species to cDNA, followed by PCR amplification of cDNA (RT-PCR). These methods are much more sensitive than Northern analysis, RNase protection assay, and in situ hybridization. In addition, they require a small amount of nucleic acids and can be used to
quantifiable gene expression (11). However, the results of quantitative RT-PCR techniques are not consistently reproducible (12). In our laboratory, Pane et al. (13) devised a noncompetitive RT-PCR procedure in which a synthetic calibrator, obtained by in vitro transcription and with the same sequence as the target mRNA, is analyzed parallel to the target samples. This procedure is highly reproducible in quantifying several specific mRNAs from healthy tissues and leukemic cells.

We set up a semiautomated dot-blot system for the comparative quantification of the RT-PCR-amplified cDNA. The method is highly efficient and reproducible. We applied this improved procedure to the quantitative analysis of aldolase A mRNA in liver biopsies from patients with chronic liver diseases at different stages. The analysis revealed a strong resurgence of the isoenzyme in HCC that distinguished HCC patients from those with cirrhosis and chronic hepatitis C.

**Materials and Methods**

**Patients**

The study was approved by the Ethics Committee of our Medical School, and informed consent was obtained from each patient. We quantified the aldolase A mRNA in liver biopsies from 12 patients with HCC on cirrhosis (9 males and 3 females; mean age, 54 years), showing a single nodular lesion with a diameter <3 cm. All of the cases were serologically positive for hepatitis C virus. The control group included 7 healthy subjects (subjects in whom histology did not reveal signs of liver disease) and 19 patients with nonneoplastic liver diseases (9 with chronic hepatitis and 10 cirrhosis patients serologically positive for hepatitis C virus). For patients with chronic hepatitis C and for four patients with cirrhosis, the tissue sample was collected during laparoscopy from a macroregenerative nodule (defined with high-grade dysplasia by pathologist); in the other six cirrhotic patients, the sample was collected from the cirrhotic tissue by fine-needle biopsy. For each group of patients the diagnosis, obtained with clinical, instrumental, and laboratory procedures, was confirmed by cytohistologic procedures. For patients with HCC, a biopsy of the surrounding cirrhotic liver was also collected. In each case, two liver samples were collected at the time of the histological analysis. One sample was used for the mRNA analysis and the other for histology, which confirmed, in all cases, the diagnosis of HCC. In four patients with HCC, the tissue samples of the neoplastic tissue and the surrounding cirrhotic tissue were obtained during surgery. The patients with chronic hepatitis C and cirrhosis were monitored for 12 months after the study to exclude the presence of HCC.

**RNA Extraction**

Total RNA was extracted from tissue as described in detail elsewhere (14). Briefly, each sample (~15 mg) was supplemented with 3 mL of guanidinium-thiocyanate buffer. The DNA was then sheared through a fine needle (22 gauge), and 300 μL of 2 mol/L sodium acetate (pH 4), 3 mL of water-saturated phenol, and 600 μL of chloroform-isoamyl alcohol solution (49:1, by volume) were added. The samples were centrifuged at 8000g for 20 min at 4°C, after which the water phase was transferred into a new tube. The RNA was precipitated twice with ethanol, and the pellet was finally washed in 700 mL/L ethanol in water, precipitated by centrifugation, dried under reduced pressure, and resuspended in sterile water. The total amount of RNA was spectrophotometrically quantified at 260 nm.

**SYNTHESIS OF RNA CALIBRATOR**

The calibrator with the same sequence as the mRNA to be quantified was synthesized as follows (15): the aldolase A cDNA, cloned in our laboratory (16), was amplified by PCR using specific primers (downstream, 5'-ATA-ACGGGCCCA GAACATTG-3'; upstream, 5'-TGACCCCCG- GACGAGAAGAA-3'). The latter primer contained, at the 5' end, the adjunctive sequence TAATACGACTCA CTATAGGGAGA to incorporate the T7 phage promoter at the 5' end of the amplified DNA (15). The amplified product, once purified by low-melting point agarose, was used as template for the RNA transcription (17). The in vitro transcription of the aldolase A cDNA yielded ~80 μg of the specific mRNA. Agarose gel electrophoresis with formaldehyde was used to verify the integrity and purity of the RNA and to exclude DNA contamination. To further verify the absence of DNA, six samples of RNA extracted from liver biopsies were amplified for the cystic fibrosis gene analysis (18); in all samples, the PCR failed to amplify DNA when we used a variety of primers usually used for the analysis of CFTR gene sequences. The mRNA was quantified spectrophotometrically at 260 nm, and scalar dilutions (300–0.03 amol) of the calibrator in tRNA solution were obtained and stored at −80°C to prevent degradation.

**AMPLIFICATION PROCEDURES AND REVELATION OF THE TARGET PRODUCT**

Both the RNA samples and RNA calibrators were reverse-transcribed into cDNA using a mixture containing, in 20 μL: 750 ng of total RNA or the RNA calibrator (diluted), 5 mmol/L MgCl₂, 50 mmol/L KCl, 1 mmol/L each deoxyribonucleotide, 2.5 units of RNase inhibitor, 0.75 mmol/L downstream primer, and 1 U of reverse transcriptase (Life Technologies). The mixture was incubated at 37°C for 1 h and at 95°C for 5 min. The PCR mixture contained 20 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 2 mmol/L MgCl₂, 0.15 mmol/L each primer, 0.2 mmol/L each deoxyribonucleotide, and 2.5 U of Taq polymerase (Perkin-Elmer Cetus) in a total volume of 100 μL. The mixture was amplified in a thermal cycler (Gene Amp PCR System 9600; Perkin-Elmer Cetus) as follows: 22 cycles of 94°C for 25 s, 58°C for 30 s, and 70°C for 30 s.

The amplified products (4 μL) were spotted, using a semiautomatic robotic dot-blot procedure (18), on a nitro-
cellulose filter (Hybond N+; Amersham). The filter was hybridized with a specific oligoprobe labeled with 32P at the 3’ end by the T4 polynucleotide kinase (Biolabs), which has the following sequence: 5’-GCGAGGA-CAAAATGGCGAG-3’. The hybridization conditions were as follows: 65 °C for 1 h, after which the temperature was left to decrease (~8 h) to 25 °C. The filter was washed for 5 min at room temperature with 5× standard saline citrate (1× = 0.15 mol/L NaCl, 0.015 mol/L sodium citrate), and then at 37 °C for 15 min with 2× standard saline citrate.

The number of amplified DNA copies was calculated by measuring the radioactivity of the labeled oligoprobe annealed, using a radioanalytic imaging system (Molecular Imager™; Bio-Rad). The radioactivity was expressed as pixel density units. The amount of amplified DNA was calculated for each sample by interpolating the result as pixel density units on a log-log chart, using a calibration curve generated with the scalar dilutions of the RNA calibrator. The amount of the specific mRNA is thus reported as amol/750 ng of the total RNA.

The optimal reverse transcription conditions had been evaluated previously on three calibrators at different concentrations, using 20 cycles of PCR amplification. We evaluated three different temperatures (37, 42, and 45 °C) and three different times (30, 45, and 60 min). The best result (in terms of linearity) was obtained at 37 °C for 60 min. To determine the limiting number of PCR amplification cycles, all of the scalar dilutions of the mRNA calibrator (0.03–300 amol) were amplified at a decreasing number of cycles starting from 30. The efficiency of PCR amplification was constant up to 24 cycles.

IMPRECISION OF THE METHOD
To evaluate the within-day imprecision of the assay, we analyzed eight times, in the same experimental series and with the same calibration curve, three RNA samples containing different amounts of aldolase A mRNA. Similarly, we tested the between-day imprecision of the method on the same three samples in six different experiments with six calibration curves constructed at the same time.

Results and Discussion
ANALYTICAL EFFICIENCY AND RELIABILITY OF THE NOVEL RT-PCR DOT-BLOT PROCEDURE
Fig. 1 shows the linearity of the assay obtained after an reverse transcription reaction for 60 min at 37 °C, and after 24 PCR cycles using the aldolase A mRNA calibration solution at different dilution points. The lower detection limit of the method was 0.03 amol, and the assay was linear up to 300 amol. The range of linearity was very dynamic, so that specific mRNAs could be measured in different biological samples in amounts ranging over a difference of five orders of magnitude (see Fig. 1). The overall analytical sensitivity of our procedure was comparable to that reported by Pane et al. (13), and by others who used either competitive (12) or multistandard (11) RT-PCR procedures. The procedure described in this study is highly specific because in addition to the two specific oligoprimer used for the PCR, an oligonucleotide complementary to the cDNA is also used to reveal and quantify the amplified product.

The within- and between-day imprecision of the method as tested on three samples with different mRNA concentrations is shown in Table 1. Again, the precision of the procedure was good (CV <15% in all instances) over a wide range of mRNA concentrations. The reproducibility of the method is comparable to that reported by others who used triple-primer PCR (19). In agreement with the latter study, our study confirms that once extracted, the RNA must be analyzed by agarose electrophoresis in the presence of formaldehyde to verify the integrity and

![Fig. 1. Calibration curve obtained from the modified RT-PCR dot-blot analysis of aldolase A mRNA at different concentrations.](https://academic.oup.com/clinchem/article/46/7/901/5641380)

<p>| Table 1. Within- and between-day imprecision of aldolase A-specific mRNA estimation by the novel noncompetitive RT-PCR procedure. |
|-----------------|-----------------|-----------------|
| <strong>Within-day imprecision</strong> | <strong>Between-day imprecision</strong> |</p>
<table>
<thead>
<tr>
<th>n</th>
<th>Mean ± SD</th>
<th>CV, %</th>
<th>n</th>
<th>Mean ± SD</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>0.042 ± 0.006</td>
<td>13</td>
<td>6</td>
<td>0.515 ± 0.063</td>
<td>12</td>
</tr>
<tr>
<td>8</td>
<td>0.153 ± 0.016</td>
<td>11</td>
<td>6</td>
<td>1.126 ± 0.079</td>
<td>7.0</td>
</tr>
<tr>
<td>8</td>
<td>3.248 ± 0.194</td>
<td>6.0</td>
<td>6</td>
<td>3.815 ± 0.435</td>
<td>11</td>
</tr>
</tbody>
</table>

*a amol/750 ng of total RNA.*
purity of the RNA. Furthermore, because the specific mRNA is quantified with reference to the total amount of RNA, it is necessary to exclude DNA contamination from the RNA. This can be done on several RNA samples by PCR amplification of a gene (in our case, several exons of the cystic fibrosis gene). Similarly, the time and temperature of the reverse transcription reaction and the number of PCR cycles must be strictly controlled. We found that a difference of 5 min or of 2 °C in the reverse transcription reaction could affect by \( \pm 20\%\) the quantitative mRNA analysis (data not shown). The good reproducibility obtained in this study also depended on the use of the robotic workstation, which limited errors and costs in the analysis of large series of samples.

The use of a calibrator with the same sequence as the target product overcomes the drawback of competitive RT-PCR procedures, i.e., the different sequences and, thus, the potentially different efficiencies of the RT-PCR amplification between the calibrator and the target mRNA (11). The calibrator used in our noncompetitive procedures has the same sequence as the target mRNA, and thus both calibrator and target mRNA are amplified with the same efficiency. In most noncompetitive procedures, however, the calibrator is obtained by cloning, which is a more complex procedure than the in vitro synthesis used in the present study.

In addition to aldolase A mRNA, we quantified a housekeeping mRNA, i.e., \( \beta\)-actin, in all tissue samples; no significant differences between HCC and the other groups of patients were obtained (Scheffé multiple comparison test). In any case, we referred the concentrations of aldolase A mRNA to total RNA because various factors such as circadian clock, hypoxia, and neoplasia could influence the concentrations of housekeeping mRNAs (20–22).

### ALDOLASE A mRNA IN CHRONIC LIVER DISEASES

The concentrations of aldolase A mRNA obtained in liver samples from the three groups of patients bearing chronic liver diseases and in the healthy controls are shown in Table 2. Aldolase A mRNA concentrations were significantly higher in patients with HCC than in the other groups of patients and in healthy subjects (\( P < 0.01\), two-sided Scheffé multiple comparison test). The aldolase A concentrations in nonneoplastic liver diseases were comparable to those obtained in healthy subjects.

### Table 2. Aldolase A mRNA concentrations in liver biopsies from patients with chronic liver diseases.

<table>
<thead>
<tr>
<th>Sample origin</th>
<th>No. of cases</th>
<th>Mean concentration(^a)</th>
<th>95% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy subjects</td>
<td>7</td>
<td>0.556</td>
<td>0.419–0.693</td>
</tr>
<tr>
<td>Chronic hepatitis C</td>
<td>9</td>
<td>0.486</td>
<td>0.296–0.676</td>
</tr>
<tr>
<td>Cirrhosis</td>
<td>10</td>
<td>0.529</td>
<td>0.455–0.607</td>
</tr>
<tr>
<td>HCC</td>
<td>12</td>
<td>4.450(^b)</td>
<td>1.760–7.145</td>
</tr>
</tbody>
</table>

\(^a\) amol/750 ng of total RNA.

\(^b\) \( P < 0.01\) (two-sided Scheffé multiple comparison test) between HCC and other groups of subjects.

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**Fig. 2.** RT-PCR dot-blot analysis of aldolase A mRNA in biopsy samples from four pairs of HCC and surrounding cirrhotic tissue. HC, hepatocellular carcinoma; C, surrounding cirrhosis.

**Fig. 3.** Aldolase A mRNA concentrations in liver biopsies from 12 pairs of HCC and surrounding cirrhotic tissue samples. The ratio (HCC vs cirrhosis tissue) of the amount of aldolase A mRNA is reported on the x axis for each pair.
We evaluated aldolase A mRNA within the HCC tissue and in the surrounding cirrhotic tissue in pairs of biopsy samples from the same patients. Fig. 2 shows an example of the results. The mRNA concentrations were clearly higher in the HCC cells than the cirrhotic tissue. Fig. 3 shows the ratio of aldolase A mRNA concentrations between HCC and surrounding cirrhotic tissue in the 12 pairs of biopsy samples; in all cases, aldolase A mRNA expression was higher in the HCC cells, with a ratio between the two values ranging from 1.2- to 21.3-fold.

Our data confirm, on a quantitative basis, the strong resurgence of aldolase A in HCC at the mRNA level. Aldolase A is expressed exclusively by fetal liver (5), not the adult tissue, and is involved in the glycolytic pathway (to split or synthesize fructose 1,6-diphosphate), whereas the adult liver expresses mainly the aldolase B isoenzyme (involved in the metabolism of exogenous fructose). In fact, serum concentrations of aldolase A have been suggested as a diagnostic tool in HCC patients (23), and our group has revealed higher concentrations of total aldolase and aldolase A in serum from HCC patients compared with patients with cirrhosis (unpublished results). The concentrations of total serum aldolase in the patients from the present study were always within the reference intervals, and no significant differences were obtained between the various groups of patients. This could reflect the different stage of HCC patients.

The various mRNA aldolase A species found in human tissues differ in the 5’ noncoding region because of alternative splicing (24), and one of these is expressed by human fetal liver cells. The primers used in the present study hybridize the region common to all the aldolase A mRNA species present in human tissues, so that results obtained from different diseases or tissues can be compared.

Because of the altered morphology of the cirrhotic liver, imaging techniques do not always lead to an unequivocal diagnosis of HCC in cases with liver lesions (1), particularly in cirrhotic patients showing nodular lesions, most of which are benign macroregenerative nodules. Similarly, cytohistology could fail to identify objectively the switch from cirrhosis to HCC (2). In this context, the analysis of specific mRNAs on the biopsy sampled for pathology could be objectively contributory, increasing the number of HCC patients who could benefit from liver resection.

We previously reported that a panel of serum biochemical markers has a high diagnostic efficiency in differentiating between HCC and cirrhosis (3). Several of these biochemical signals (i.e., α-fetoprotein, lactate dehydrogenase, and γ-glutamyltranspeptidase isoenzymes) are specifically produced or overexpressed by neoplastic cells. Therefore, quantitative analysis of their mRNA species can be added to the analysis of aldolase A mRNA as a contributory tool for the diagnosis of HCC.

In conclusion, the noncompetitive RT-PCR procedure for aldolase A mRNA quantitative analysis described here is highly efficient, reproducible, and with small cost, can be extended to the analysis of other mRNA species. In addition, the quantitative analysis of aldolase A mRNA can, together with cytohistologic procedures, play a role in the diagnosis of HCC.

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