Comparative analysis of invasive and non-invasive methods of liver fibrosis measurement in liver diseases of various etiologies

Ph.D. Thesis

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I. INTRODUCTION

Chronic liver diseases pose a significant health problem not only in Hungary but worldwide. According to the WHO data published in 2014, in Hungary the mortality rate of liver cirrhosis was 27.21 per 100 000 patients, meaning we were ranked 36th among the 172 countries studied. The WHO data also showed that in 2012 liver cirrhosis was the cause of death in 3 900 cases, being therefore the 7th most frequent death cause in Hungary.

Numerous etiological factors may play role in the development of chronic liver disease (virus, autoimmune disease, metabolic disorder, toxic agent, genetic disorder, etc.). In the majority of cases the disease has no or only poor symptoms, often making diagnosis possible only in the advanced stage. Regardless of etiology, chronic liver diseases have a common course with the presence of inflammation in the liver, which over time is followed by increase in connective tissue – fibrosis – then by structural distortion and remodeling – cirrhosis. It is most likely that hepatocellular carcinoma (HCC) will develop in a cirrhotic liver, with an incidence of 3-5% per year. Early recognition of chronic liver disease is essential for both patient and society. For the patient, detection of the disease and successful treatment help to maintain health, quality of life and everyday activities. From the viewpoint of society, early diagnosis can prevent severe
health problems that may arise from infection (cirrhosis and its consequences: varix bleeding, vascular and parenchymal decompensation, etc.) resulting significant savings in health care costs.

In case of chronic, diffuse liver diseases, the most important information for judgement of course and prognosis as well as choice and effectiveness of therapy include recognition of the stage of disease, confirmation of the presence and degree of fibrosis, and the determination of fibrotic changes. Nowadays these are markedly the most timely objectives in the treatment of chronic viral hepatitis, especially chronic hepatitis C (CHC).

In the past 50 years the gold standard for the diagnosis of fibrosis was liver biopsy. However, due to its invasive nature and the possibility of sampling errors as well as intra- and interobserver errors, the necessity of taking biopsies in the routine diagnostics of chronic liver diseases is being questioned. At the same time, an indisputable advantage of the procedure is that a number of molecular, immunohistochemical studies may be performed from biopsy samples and also quantitative assessment of fibrosis is made possible by digital morphometric analysis (DMA).
Based on the above, there have recently been many important advances in non-invasive methods for the diagnosis of liver fibrosis. These include blood-based markers and a combination of these – such as APRI (AST-to-Platelet Ratio Index) – as well as imaging and scanning techniques (e.g. Transient Elastography - Fibroscan). Their value and accuracy are currently under discussion.

Among the aims of our present work was to explore the possibilities of determining fibrosis in liver diseases of different etiologies and to compare the efficiency of invasive and non-invasive techniques.

There are several liver diseases in which microRNAs (miR) – these small, non-coding RNA molecules, which are generally negative regulators of gene expression – exhibit characteristic changes. It is assumed that their expression levels also change during fibrosis and are thus indicative of the degree and progression of fibrosis. We therefore wished to determine whether tracking the changes in miR expression would increase the accuracy of histopathological liver fibrosis assessment.
II. OBJECTIVES

Based on the above, our aim was to assess the value of and draw comparisons between "traditional" invasive and non-invasive methods, as well as new testing procedures during liver fibrosis of different etiologies. We therefore sought to answer the following questions:

1. What are the observable correlations between the data of traditional semi-quantitative fibrosis determination method (Metavir) and digital morphometric analysis (DMA) during the analysis of liver needle biopsy specimens?

2. Can digital morphometric analysis help to make a more accurate assessment of fibrosis stage?

3. What correlations can be found between fibrosis stages determined histologically and assessed by non-invasive approaches (Liver stiffness (LS) measured by Transient elastography (TE), as well as APRI)?

4. Is accurate assessment of liver fibrosis by means of different methods influenced by degree of steatosis, sample size (number of portal tracts), degree of simultaneous inflammation, or gender?

5. Does the intrahepatic expression of miRs showing alterations in HCC (miR-21, miR-122, miR-214, miR221/-
222, miR-224) correlate with the degree of fibrosis and ALT level, an indicator of liver disease?

6. Is the relationship between fibrosis stage as determined by traditional semi-quantitative method (Metavir), LS value and the various miR expressions influenced by the etiology of chronic, diffuse liver disease?
III. METHODS

Patients

Liver biopsy samples of 96 patients with chronic liver diseases from Hepatology Centers in Hungary (53 females, 43 males; age: 15-67 years, mean: 46.05 years) were included in DMA examinations. Molecular analysis was performed in 52 cases.

Histology

Biopsy samples were processed according to routine pathological procedures. These samples were cut into 3-4-μm thick sections and stained with hematoxylin-eosin and picrosyrius red to highlight the connective tissue.

Determination of fibrosis

Histological staging and TE examination were applied to determine the severity of fibrosis. Histological staging was performed by two pathologists using the Metavir scoring system from stages F0 to F4, with stage F0 indicating no fibrosis and stage F4 representing cirrhosis. The non-invasive TE was carried out using FibroScan 502 (Echosens, Paris, France), with low LS values reflecting no or mild fibrosis and high LS values implying advanced fibrosis or cirrhosis. The time elapsed between date of
histological sampling and date of LS measurement was a maximum of 3 months, with an average of 1.5 months.

**Quantitative digital morphometric analysis**

Liver biopsies were stained with Sirius red. Quantitative analyses of the tissue samples digitalized by Mirax Midi slide scanner equipped with a 20x Zeiss Plan-Apochromat objective and Marlin F146C camera (3DHistech Ltd, Budapest, Hungary) were performed. Areas of the whole sample and the designated fibrotic region were measured to calculate CPA. Measurements were made under preset, standardized circumstances using Leica QWin V3 morphometrical software (Leica Microsystem Imaging Solution Ltd., Cambridge, UK).

**Biochemical examinations**

APRI score (based on ALT, AST, platelet count) was determined at the time of liver biopsy (APRI= AST/ AST ULN/platelet count); (10⁹/l)×100).

**RNA isolation**

RNA was isolated from several 3-4-μm thick sections using the RNeasy FFPE Kit (Qiagen, Venlo, Netherlands) according to the manufacturer’s instructions with modifications for copurification
of miRNA. Traces of genomic DNA were eliminated using Turbo DNase digestion (Ambion, Austin, TX, United States).

**Reverse transcription (RT) and quantitative (q)PCR**

Expression of individual miRNAs was determined using the following TaqMan MicroRNA Assays (Life Technologies of Thermo Fisher Scientific Inc., Waltham, MA, United States). Briefly, RT reaction was carried out using the TaqMan MicroRNA Reverse Transcription Kit in a final volume of 7.5 μL containing 10 ng total RNA. The qPCR was performed using TaqMan Universal PCR Master Mix No AmpErase UNG in a final volume of 10 μL containing 0.65 μL RT product. The amplification reaction was run in triplicate on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems of Thermo Fisher Scientific Inc.). Relative expression was calculated by the 2-ΔΔCq formula, applying miR-140 as the most stable reference determined by the NormFinder application and normalized to the median ΔCq value of F0 liver samples.

**Statistical analysis**

The differences between fibrosis stages F0-F4 were analyzed with a non-parametric Kruskal-Wallis analysis of variance and
median test using STATISTICA software, version 9.1 (StatSoft Inc., Tulsa, OK, United States).

Correlation analyses between miRNA expression and fibrosis stage, LS values, and ALT levels were performed with a non-parametric Spearman rank order correlation using GraphPad PRISM software, version 5.01 (GraphPad Software Inc, La Jolla, CA, United States). A $P$ value of 0.05 was set as the threshold for statistical significance.

For DMA correlations between the noted variables were determined using Spearman rank's correlation test. Differences were considered significant when $P<0.05$. All statistical analyses were performed using Statistica 9.0 (StatSoft Inc. Tulsa, OK) software program.

IV. RESULTS

Analysis of samples

Significant positive correlation was verified between the results of the histological analyses and the non-invasive methods used to evaluate liver fibrosis in case of all 96 patients. The strongest correlation was found between Metavir scores and DMA then, in descending order, between LS values and Metavir scores, LS
values and DMA, LS values and APRI scores and finally between Metavir and APRI scores, respectively.

**Evaluation according to fibrosis stage**

Significant positive correlation was observed between LS values and the results of DMA and strong positive correlation was also detected in the advanced stages of liver fibrosis (F3 and F4).

**Evaluation according to number of portal tracts**

A substantial, though relatively weak correlation was found between LS values and results of DMA in cases in which less than 10 portal tracts were present, whereas the correlation was strong in cases involving a large number of portal tracts. Significant positive correlation was notable between LS values and the results of DMA in cases showing advanced fibrosis (F3 and 4) with both low (<10) and high (≥ 10) numbers of portal tracts, but the correlation was stronger in cases of large sample sizes.

**Evaluation according to sex**

The correlation between DMA and Metavir score was the same in both male and female patient groups, being similar between LS values and Metavir scores.
Role of fat content of liver tissue
No differences were detectable between correlations of DMA and Metavir score, LS values and Metavir score as well as LS values and results of DMA in cases of low-grade (Grades 0, 1) and high-grade steatosis (Grades 2-3).

Evaluation of results in patients with chronic hepatitis C
The samples of 53 patients suffering from chronic hepatitis C were analysed separately, with the finding that correlation was the strongest between LS values and Metavir score. Similar correlation was found between LS values and results of DMA, and LS values and APRI score.

Role of Histological Activity Index (HAI)
Out of 53 patients with chronic hepatitis C, 24 demonstrated necroinflammation. (HAI levels >6) The correlations between the various methods used for evaluation of fibrosis were examined according to HAI level. No differences were found between DMA and Metavir score in the two groups. Furthermore, no relevant differences were observable between LS values and the Metavir score in cases showing low (HAI ≤ 6) or high (HAI >6) inflammatory activity.
Determination of fibrosis

The METAVIR scoring system allowed unambiguous determination of the fibrosis stages in each tissue sample. In contrast, the non-invasive LS measurement showed a wide range of values and when matched with the corresponding METAVIR stages, an overlap between the neighbouring ranges was observable. This was predominantly manifest in cases of LS value ranges that corresponded to fibrosis stages F0-F3. In addition, the LS value ranges showed slight variances between the various etiology groups. Yet, a highly significant correlation was found between the gradually increasing LS values and fibrosis stage.

Expression of individual miRNAs in relation to Metavir stage

Expression of individual miRNAs showed deregulated patterns in stages F1-F4 in comparison to stage F0, but the observed differences, except for one case, did not reach the set threshold for statistical significance. The exception was miR-122, in which case the expression in stage F4 was decreased as compared with stage F0. The expression differences were close to reaching statistical significance in two cases: miR-122 between stages F1 and F4 and miR-214 between stages F2 and F4.
When looking at the expressional patterns of individual miRNAs, in general, the levels were lower in stages F1-F4 in comparison to F0, showing an increasing tendency in case of miR-214 from F2 to F4, miR-222 from F1 to F4 and miR-224 from F2 to F4. Nevertheless, the differences in the three etiological groups did not reach the set threshold for statistical significance.

Correlation of miRNA expression with fibrosis and ALT values
In relation to miRNA expression and fibrosis, miR-122 and miR-221 showed negative correlation with METAVIR stage and miR-122 was found to correlate negatively and miR-224 positively with LS values. Furthermore, a positive correlation of miR-21 was found between miRNA expression and ALT levels.
V. CONCLUSIONS

1. This is the first study in Hungary involving such a large number of cases, using digital morphometric analysis to compare invasive and non-invasive fibrosis markers in chronic liver diseases of various etiologies.

2. The values obtained by means of digital morphometric analysis (DMA) correlated well with the Metavir score and the results of the non-invasive methods used to evaluate liver fibrosis, primarily with the LS value. Accordingly, DMA may be a useful additional tool to determine histopathological fibrosis stage.

3. The non-invasive methods for the evaluation of liver fibrosis stage, - LS and APRI - , showed good correlation with the histopathological techniques. LS showed stronger correlation than APRI.

4. The accuracy of both DMA and non-invasive techniques increased in the advanced fibrosis stages.

5. The correlation between fibrosis determination measured by invasive and non-invasive techniques was stronger with the increase in biopsy sample size.

6. The presence and degree of steatosis, gender, and degree of histologically determined inflammatory activity (HAI) did
not influence the results of DMA or non-invasive assessments of fibrosis.

7. We are the first to show that the expression of miR-122 was decreased in chronic liver diseases of various etiologies, depending on the severity of fibrosis. Three microRNAs, which also play role in hepatocarcinogenesis (miR-122, miR-221 and miR-224) showed correlation independent of etiology with the values of Metavir and/or LS. From the three, miR-122 seems the most likely to become a future useful molecule in the diagnosis of fibrosis.

8. miR-21 showed positive correlation with ALT level, an indicator of the degree of inflammatory activity.

9. Regarding the various etiological groups, no significantly different patterns of miR expression were observable.
VI. LIST OF PUBLICATIONS

Publications related to the thesis


Publication not related to the thesis

1. Lendvai Gábor, Jármay Katalin, Karácsony Gizella, **Halász Tünde**, Kovalszky Ilona, Baghy Kornélia, Wittman Tibor, Schaff Zsuzsa, Kiss András: Elevated miR-33a and miR-224 in steatotic chronic hepatitis C


4. Horváth Gábor, Tolvaj Gyula, Halász Tünde, Stotz Gyula: The Role and the Possibilities of Natural Interferon Treatment for Chronic Hepatitis C: Our Experiences with Natural Interferon Treatment for Patients Debarred from Combined Antiviral Therapy due
to the STOP Rule HMJ (Hungarian Medical Journal) 2007, 1, 331-339.

