Review Article

Current Strategies for Quantitating Fibrosis in Liver Biopsy

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Abstract

Objective: The present mini-review updated the progress in methodologies based on using liver biopsy.

Data Sources: Articles for study of liver fibrosis, liver biopsy or fibrosis assessment published on high impact peer review journals from 1980 to 2014.

Study Selection: Key articles were selected mainly according to their levels of relevance to this topic and citations.

Results: With the recently mounting progress in chronic liver disease therapeutics, comes by a pressing need for precise, accurate, and dynamic assessment of hepatic fibrosis and cirrhosis in individual patients. Histopathological information is recognized as the most valuable data for fibrosis assessment. Conventional histology categorical systems describe the changes of fibrosis patterns in liver tissue; but the simplified ordinal digits assigned by these systems cannot reflect the fibrosis dynamics with sufficient precision and reproducibility. Morphometric assessment by computer assist digital image analysis, such as collagen proportionate area (CPA), detects change of fibrosis amount in tissue section in a continuous variable, and has shown its independent diagnostic value for assessment of advanced or late-stage of fibrosis. Due to its evident sensitivity to sampling variances, morphometric measurement is feasible to be taken as a reliable statistical parameter for the study of a large cohort. Combining state-of-art imaging technology and fundamental principle in Tissue Engineering, structure-based quantitation was recently initiated with a novel proof-of-concept tool, *q*Fibrosis. *q*Fibrosis showed not only the superior performance to CPA in accurately and reproducibly differentiating adjacent stages of fibrosis, but also the possibility for facilitating analysis of fibrotic regression and cirrhosis sub-staging.

Conclusions: With input from multidisciplinary innovation, liver biopsy assessment as a new "gold standard" is anticipated to substantially support the accelerated progress of Hepatology medicine.

Key words: Chronic Liver Disease; Cirrhosis; Hepatic Fibrosis; Liver Biopsy; Quantitative Assessment

Introduction

Chronic liver disease (CLD) represents a significant public health problem world-wide. The current major ones, such as viral hepatitis and nonalcoholic fatty liver disease, were estimated to have a collective morbidity of up to 20% of the general population.[1-3] Without effective management, CLDs normally share a common pathological process of progressive fibrogenesis, resulting in increasing risk of cirrhosis and liver cancer. For example, 15%–40% patients with chronic hepatitis B patients may progress to cirrhosis or hepatocellular carcinoma during their lifelong time.[4,5] The fundamental aim of CLD treatment is to halt or even reverse the development of hepatic fibrosis and cirrhosis. Thus, it is invaluable to accurately diagnose and monitor hepatic fibrogenesis in the individual patient, for the purpose of timely and effective prevention, treatment and prognosis of CLD.

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With recently mounting advances in therapeutics of CLD, particularly the chronic viral hepatitis, it has been verified that regression of fibrosis or even cirrhosis can be attained with long-term effective causative therapies.[6,7] Accompanying the evolving treatment strategies, the needs for precise, accurate, and dynamic evaluation of hepatic fibrosis and cirrhosis are becoming more urgent than ever in the areas of both hepatic clinical treatment and the relevant drug development. It is now a burning question regarding how to achieve the Holy Grail of hepatic fibrosis assessment with the following characteristics,[8,9] such as, being CLD specific; providing data less influenced by clinical biochemical parameters of liver or the other organs; having precise, sensitive, and dynamic discriminability for analyzing adjacent fibrosis stages, progression and regression in any CLD; involving relevant etiological mechanisms, and being efficient to perform assessment. Till now, there have been amounts of methodologic reports

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addressing this question. They can be generally classified into the invasive and noninvasive approaches. As there have been recently several systematic reviews on the noninvasive approaches,[10,11] the present mini-review focuses on updating the progress in methodologies based on using liver biopsy.

Value of Histopathological Information for Hepatic Fibrosis Assessment

Major causes for chronic liver injury consist of viral hepatitis, alcoholic and nonalcoholic steatohepatitis, hepatic biliary diseases, autoimmune hepatitis, drug-induced hepatitis, and metabolic genetic liver diseases. Extracellular matrix (ECM) over-accumulates, remodels and forms into fibrous scar in liver tissue that is iteratively injured and suffers chronic inflammation of diverse etiology.[12] Fibrous scar changes the physiological architecture of hepatic tissue; thus undermines the microenvironment which is fundamental for liver cells to function normally well. Relevant clinical phenotype or syndrome will develop and become apparent accordingly with the evolving patterns of fibrous distribution.[13,14] Since 1980s, there has been great advances in identifying the biomolecular process underlying liver fibrogenesis;[14] in addition to basic knowledge, it was also revealed in *in vivo* models that different injuries of CLDs with distinct or even the same etiology can cause different processes of inflammation, and thus diverse histological patterns and progression of liver fibrosis, which in turn result in various phenotypes of liver functional performance and disease prognosis.[15,16] Most of the above knowledge was obtained from experimental research using animal or cell culture models. However, hepatic fibrogenesis in clinical CLD usually takes a remarkably longer evolution duration; for example, it takes about 15–20 years for the occurrence of cirrhosis of chronic hepatitis B progressing from its initial stage of infection;[17,18] in addition, there exist long-lasting, dynamic, and multifactor-interlocked interactions between various etiological elements and patient's individual phenotypic or genetic conditions. The involved pathological process is too complicated and hardly possible for any experimental model to faithfully recapitulate it. Therefore, it is rational that research data from experimental studies are virtually lack of potency for substantively solving clinical issues regarding treatment and management of fibrosis. This may somehow explain why there is still hardly any antifibrosis treatment clinically approved and licensed as yet,[19] even if significant progress in the identification of potential therapeutic targets has been achieved in the laboratory.

In current clinical scenario, the results of liver function biochemistry and etiology testing are mainly referred to for therapeutic decision. However, these data cannot provide any direct information regarding the exact landscape of a specific pathological progression, which is invaluable for making accurate treatment decision and prognostication of the clinical situation of a particular organ. So which approach can, to a large extent, provide such direct information? One of the fundamental theories rooted in Tissue Engineering Research, which is comprehensively observed and confirmed in the Research of Developmental Biology, is that there exists close association between the physical structure and the functional behavior within any organisms.[20,21] Translated into the terms of biomedicine, from the particular view of fibrosis-dominant disease, the postulation could be that there exist ways of correlation between the information of fibrotic structural pattern and pathophysiological function phenotype of the fibrotic tissue. Indeed, this point has been suggested by the findings in clinical practice, that is, the characteristics of the histological pattern are distinct among CLDs of different etiologies.[13] At organ level, while the hepatic fibrosis caused by biliary disease normally has various display among different lobes, hepatic fibrosis of chronic viral hepatitis, alcoholic and nonalcoholic steatohepatitis, and autoimmune liver disease distributes in a relatively uniform way. Thus, histopathological conditions of CLDs of the latter etiologies can be rationally represented by liver biopsy sampling with the appropriate adequacy following the relevant practice guideline.[22,23] In observation at tissue cellular level, characteristics of fibrous ECM, such as morphology, quantity, and distribution trend, always change along the lines of different etiologies, inter-stages and intra-stages of the same etiology, and patient individuals.[14,24] For example, it was found that while the fibrotic pattern normally starts and spreads from portal tract to surrounding vascular in chronic viral hepatitis; in alcoholic and nonalcoholic steatohepatitis, fibrous ECM is dominantly accumulated in perisinusoidual and pericellular location. In addition, studies in natural history of chronic viral hepatitis indicated that the progression of pathophysiology conditions of patients with chronic hepatitis B is often accompanied with the histological changes of hepatic fibrosis instead of necroinflammation.[25] Thus, changes of fibrotic structure potentially could be more reliable and accurate for monitoring and predicting the pathophysiological conditions of CLD than the other clinical parameters.

Conventional Histologic Assessment

Clinical hepatopathologists perceived the association between histology and pathophysiology more than 50 years ago.[26] Now-a-days in terms of evidence-based medicine, among the achievements of pathological diagnostic systems established for chronic disease of other organs, the histological evaluation system built-up for CLD, particularly the chronic viral hepatitis, is highly outstanding for its "gold standard" quality of widely-approved clinical application and its critical role in guiding practice.[27] They proposed that change of fibrosis pattern could be more reliable for properly analyzing the disease condition at its chronic stage,[23,28] which was verified by mounting clinical studies that there is definite association between the progression or regression of hepatic fibrosis and the conditions of out-of- or under-control of the CLD.[17,26,29] But as for the information of fibrosis that can be used for specific, precise and accurate evaluation of the CLD progression, how to identify and derive them

from the other tissue information? Given the evidence above, the strategy that can completely and precisely collect the clinical relevant architectural information of fibrous ECM (i.e., architectural fibrosis marker) could be suitable to address the requirement. But have we got this strategy?

There have been numerous methodologies reported for the measurement of hepatic fibrosis using noninvasive and invasive technologies. Comparison of their fundamental strategies was summarized in Figure 1. In addition, considering current validated status of these technologies in clinical settings, there have been suggested various preferences for their application in distinct fibrotic situations [Figure 2].[9] These relevant applications are anticipated to evolve more rapidly and comprehensively, especially regarding how to define and monitor the regression of fibrosis and cirrhosis, owing to the increasing endeavors dedicated for them.

Current commonly available approaches of noninvasive technologies mainly include blood test and liver stiffness (LS) measurement using transient elastography.[10,11] Noninvasive approaches have been attaining more interest in real life clinical practice, primarily due to their intrinsic advantageous applicability for screening and serially monitoring some special subgroups of patients. However, data generated by these noninvasive approaches do not contain any component of fibrotic architectural information. While blood test is an indirect measurement specifically designed to characterize the serum biochemical changes during different stages of liver fibrosis, LS is a novel parameter measured directly in the liver.[30] Though LS is an excellent surrogate marker of advanced fibrosis and cirrhosis, it is not only linked to hepatic fibrosis. As a matter of fact, it is also influenced by other clinical settings such as hepatic congestion, hepatic vessel pressure, mechanic cholestasis, and hepatic inflammation.[30] Therefore, its clinical relevance should always be translated in the context of etiology, imaging and laboratory findings. This somehow explains why the range of appropriate application and underlying meaning of these technologies are still being explored since they are entirely different parameters from the well-validated histological items. More longitudinal prospective studies are needed for the purpose that the noninvasive approaches could finally be substantially taken as an alternative surrogate for predicting CLD outcomes.

The invasive assessment approaches are the ones performed with sampling liver tissue. Conventional histological evaluation systems, e.g., the categorical assessment algorithm, belong to this subtype. Due to the ability to directly provide morphological information of injured liver tissue, of which the clinical relevance has been validated in viral hepatitis, these approaches are regarded as the "gold standard" reference for fibrosis assessment in both clinical practice and studies, which supposedly may not be replaced by noninvasive approaches in a foreseeable future. Since 1980s, the categorical histological assessment such as Knodell, Metavir, and Ishak. systems came to appear, becoming well accepted in clinical applications.[31-33] These systems share the same principle of using semiquantitative scoring algorithm that assigns serial numerals to different histological patterns. Note that these numerals are categorical labels other than quantitative variables, so that what they indicate is neither a degree nor amount of fibrosis, but the various pattern of fibrous architectural changes in liver tissue.

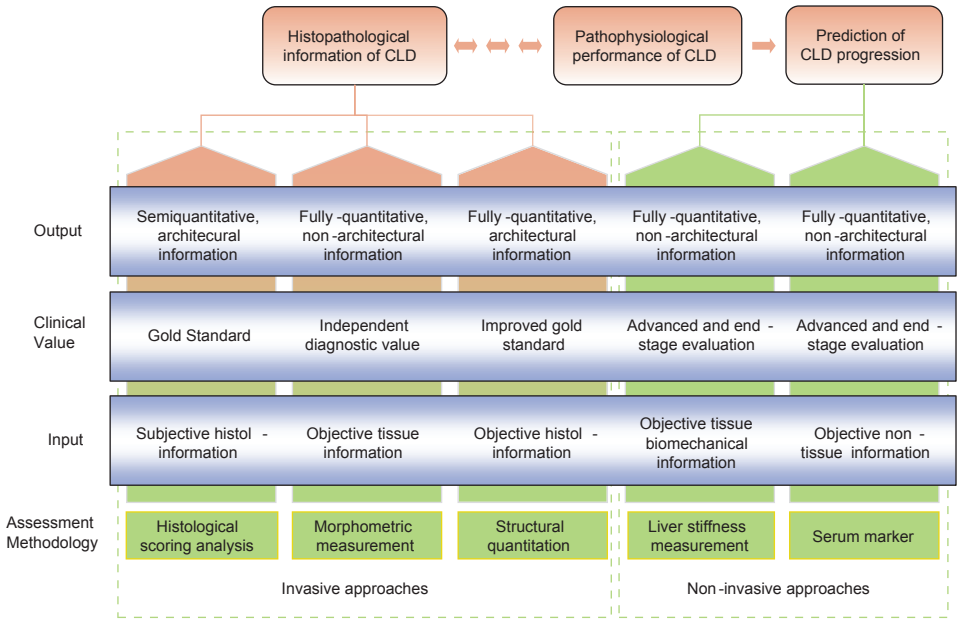

Figure 1: Comparison of current major approaches for hepatic fibrosis assessment and their roles in predicting progression of CLD (CLD: Chronic liver disease).

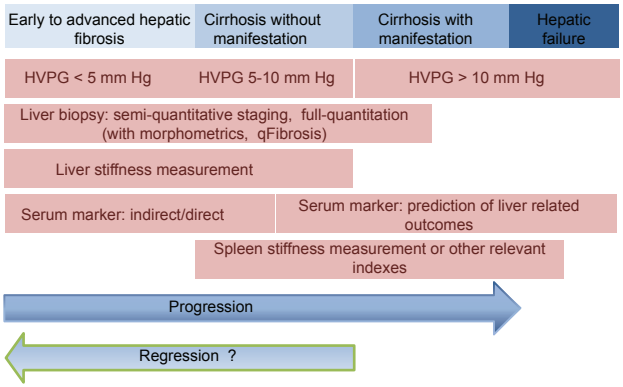

Figure 2: Application of the current technologies for assessing hepatic fibrosis at different stages of CLD (HVPG: Hepatic vein pressure gradient; CLD: Chronic liver disease).

Because when these systems were established, there still lacked effective treatment for preventing CLD progression, they had not included the diagnostic pattern of indication for fibrosis regression. That is to say, the objective to which the systems can be properly applied is the ones of progressing CLD; improper evaluation may occur if using these patterns for diagnosis of regressing CLD. In addition, cirrhosis was simply assigned one or two stages in these categorical systems, since in 1980s cirrhosis almost equaled to inexorable death of end-stage liver disease. However, understanding of the pathophysiological changes underlying cirrhosis has been increasingly systematic, and regression of cirrhosis of viral hepatitis by long-term antiviral therapy has been verified in several multicenter clinical trials of large cohort,[6,34] so that the simple one- or two-stage definition of cirrhosis is obviously outdated to adequately reflect the complexity and kinetics of cirrhotic condition. Dynamic and etiology-based characterization of cirrhosis pathological process, including precisely defining cirrhotic progression/regression and discriminating cirrhosis substages, is now in a great need for monitoring the efficacy and facilitating accurate therapeutic decision.[9,35]

Conventional histological assessment systems currently used in clinical practice normally employ a descriptive semi-quantitative way for staging fibrotic evolution. The procedure of assessment can be conceptually summarized as that, through reading by individual pathologist, the confluent and complex information of CLD histology is minimized into several serial numerical scores when reaching the physician. In fact, although description of histological features always accompanies the reported ordinal digits, physicians can be more impressed by virtually nothing else but one of the four (Knodell), or five (Metavir), or seven (Ishak) categorical labels. Consequently, the semi-quantitative systems are widely complained against their inherent subjectivity and loss of information integrity, which result in the major issues such as observer variability and sampling error in its application, even performed strictly following the practice guideline for liver biopsy. For example, according to a study by Regev *et al.* for observing the variances of chronic hepatitis C liver biopsy assessment,[36] the basic frequency of sampling error of inter-lobes can be 9.7%, at least one-stage difference reading can be 24.2%, and the under-diagnosis of cirrhosis can happen in 14.5% patients. These digits may be worth attention of clinical researchers in liver biopsy assessment, due to their indicative information of system bias. Ratios of histological change reported being around these numbers have to be interpreted with caution, since the numbers may be possibly attributable to sampling variances rather than the effectiveness of treatment intervention *per se*. Misinterpretation of histological data can become even more serious when the size of biopsy sample lacks adequacy, that is, below the standard of ≥20–25 mm in length and ≥1.2 mm in width with ≥11 complete portal tracts.[37] It was repeatedly demonstrated that the shorter the sample is, the more possibly the fibrosis of it tends to be underestimated.[37,38] Generally speaking, although previous report on establishment of these systems claimed these methodologies both reproducible and accurate, it has been widely recognized that their pragmatic performance in assessment of hepatic fibrosis can be neither of the above positions.

Morphometric Assessment

To overcome the limitation of being semi-quantitative, since the end of 1990s, researchers have been trying to fully quantitate fibrosis of liver biopsy based on the strategy of morphometric assessment. The methodology basically includes fibrous collagen-specific staining, digital imaging tissue section, and computer assist digital image analysis. Collagen proportionate area (CPA) measurement is a typical morphometric approach. It calculates the area ratio of fibrillar collagen (FC) (ideally stained with Picro-Sirius red) to its correspondent tissue section.[39] CPA generates continuous variables in a theoretically objective way, although in a technical aspect, the measurement consistence still has to suffer greatly from the variances of staining procedures, operator experience, and software of image analysis. It also needs to note that CPA value is significantly vulnerable to sampling size variances. It is easy to understand that fibrosis amount in different subareas of even the same sample normally cannot distribute evenly. Therefore, it is suggested that CPA should not be directly used for evaluation of fibrosis in an individual patient, but may be taken as a statistical parameter for a large cohort.[40]

The fully quantitative methodology, which CPA employs, makes it feasible for researchers to detect the fine changes of fibrosis amount in liver tissue. CPA has been used in numerous clinical studies of CLD fibrosis with different etiologies underlying since it is supposed to be helpful for substaging some conditions of hepatic fibrosis and cirrhosis. Although recently there is increasing interest in exploring CPA's role in fibrosis measurement of alcoholic and nonalcoholic liver diseases,[41] most studies till now were performed with liver biopsies of chronic hepatitis C or B.[39,42,43] Among these studies, while some reported that CPA values correlated well with scoring of all histological

categories from mild to advanced fibrosis; the others found that change of CPA value was not sensitive enough to discriminate the mild and significant fibrosis, but almost linearly associated with the progression of advanced to end-stage fibrosis. The CPA performance in our recent studies of drug-induced fibrosis rat model and chronic hepatitis B liver biopsies agreed with the later ones.[44]

Regarding using CPA for diagnosis and substaging of cirrhosis, a significant correlation between the values of CPA and LS or hepatic vein pressure gradient (HVPG) within the compensated phase was suggested by several recent studies;[24,42,45] the underlying rationale for it might be, since cirrhosis is histologically defined as a pathological condition that tissue architecture is distorted by diffuse fibrous ECM (mainly consisting of FC I and III), the amount of FC might start to play a major role in predicting progression of cirrhosis before the stage of advanced portal hypertension (i.e., HVPG $\geq$ 12 mmHg); so that during the course of compensated cirrhosis, HVPG could be influenced significantly by FC quantity, which is what the values of CPA and LS mainly indicate. Whether CPA could be used as another prognostic surrogate marker of the dynamic cirrhotic conditions is worth comprehensive investigation in more clinical settings.

Though CPA has the advantage of being fully quantitative and probably an independent marker in substaging compensated cirrhosis and prognostication of decompensation complications, the information it provides is limited to the amount of fibrosis without any spatial cue. It might be some sort of "resource-wasting" if tissue specimens are taken just for collecting the information of fibrosis amount since the tissue samples *per se* contain far more fibrosis-relevant information beyond this factor only. In addition, as a matter of fact, CPA does not solve the major drawbacks of conventional histological assessment, because it virtually takes a distinct underlying strategy from the one that histological assessment systems are commonly based on, emphasizing the morphological patterns of fibrotic changes. Therefore, on one hand, study of CPA needs to move on further exploring and validating its potential role in CLD applications; on the other hand, innovative methodologies that can substantially improve the quality of current "gold standard" are still on great demand.

Structure-based Quantitation Assessment

The conceptual meaning of hepatic fibrosis in CLD contains the entire information of pathologically accumulated fibrous ECM, which is basically composed of biochemical *vs.* physical, and temporal *vs.* spatial elements. Hepatopathologist innovators of conventional histological assessment systems perceived (with their professional intuition) the mechanistic essence underlying the specific CLD conditions. The conventional systems established along this line were afterward validated of their efficacy in clinical settings. Such an experience may somehow inspire us that the relatively complete information of fibrotic tissue with particular underlying pathological conditions could hold the potential to properly represent the pathophysiology state of the tissue. This point would rationally depend on the informative confluence of three elemental factors, that is, fibrous component, relevant morphology and quantity, to define whether the fibrotic histological information being complete or not.

Fibrillar collagen is commonly the major component of fibrous ECM in CLD of different etiologies, or even different organs of fibrotic disease. Then quantification of the architectural pattern of FC can be privileged as the target for study of the histological change of hepatic fibrosis at a particularly defined pathological condition. So how? First is to acquire the relatively complete histological information of FC. There are several ways to identify the existence of FC. Collagen specific color-metric stain and immunohistochemical stain in tissue section are widely used in both clinical and experimental laboratory; however, considering the detection quality of standardization and sensitivity, these techniques are not the optimal solutions for *in situ* morphological and quantitative measurement. Moreover, it needs a lot of preparation for the staining procedures, which is rather lousy and prone to batch-to-batch inconsistence, so that hard to be carried out routinely in clinics.

Recent advances in biophotonics application in Tissue Engineering provided unique techniques for FC detection. Since the first report of nonlinear imaging of biosamples in 1990,[46] application of multiphoton microscopy, such as the second harmonic generation (SHG) microscopy, has been actively explored in biomedical imaging research.[47-50] The fundamental physical principle of SHG imaging in FC molecule is illustrated in Figure 3. SHG imaging of FC has distinct advantages compared to other imaging modalities,[51] including that tissue samples for imaging can be label-free since SHG signals are generated due to the intrinsic

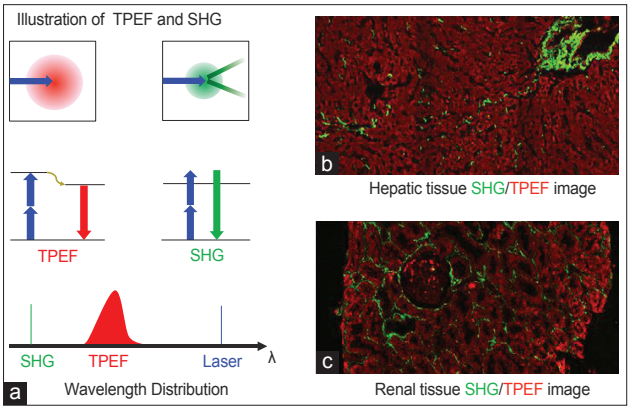

Figure 3: Basic physical principle of SHG/TPEF imaging (a) and its application in hepatic (b) and renal (c) tissue fibrosis imaging in tissue images (b and c), green signal shows the fibrillar collagen imaged by SHG; red signal show the tissue cells imaged by TPEF. (SHG: Second harmonic generation; TPEF: Two-photon excited fluorescence).

noncentrosymmetric structure of FC molecule; the penetration depth can reach to 200 μm because excitation wavelength for SHG is usually adjusted in the near infrared range; and the known excitation and emission spectral signatures of SHG allows easily and sensitively separating signals of FC from other fluorophores. Due to the unique strength of SHG in FC imaging, combining another nonlinear optical technology of two-photon excited fluorescence (TPEF) for tissue cell imaging, some research groups tried to use SHG/TPEF in animal models of liver fibrosis and validated its technical performance and feasibility for hepatic fibrosis imaging.[50,52] The first reported clinical application of SHG/TPEF for CLD hepatic fibrosis measurement was by Guilbert *et al*. in 2010.[53] The study comprehensively validated the technical feasibility of SHG/TPEF using 107 tissue specimens of mixed etiology. Although employing the special imaging technology, it took CPA for fibrosis analyses, so that in some sense it could be regarded as a technical report rather than a study for improving the accuracy of fibrosis assessment modality *per se*.

Focusing on improving the value of histological assessment, structure-based quantitation (SBQ) was recently initiated by our study.[44] The fundamental principle of SBQ is to fully quantitate the features of the histological pattern of FC, e.g., the number, length or texture of FC. Combining the strength of SHG technology, SBQ translated the principle of Metavir fibrosis scoring into a new tool of fully-quantitative algorithm, named qFibrosis, which was then demonstrated to be able to automatically, accurately and reliably score hepatic fibrosis and cirrhosis in both animal model and liver biopsies of chronic hepatitis B.[44]

The design of *q*Fibrosis index encompassed three key morphological phenotypes of common pathological interest and had them quantified into three subindices by measuring the spatial parameters of FC within each individual location. *q*Fibrosis can significantly identify differences between all Metavir fibrosis stages, indicating that it can faithfully and reliably recapitulate Metavir scoring principle. Within the framework of histopathological categorization, *q*Fibrosis provides scores of continuous variable attributed to its inherent full-quantification capability; thus, it could potentially be used to precisely reflect the dynamics of fibrosis/cirrhosis progression or regression.

In addition, tested with different sizes of biopsy samples, *q*Fibrosis unprecedentedly and significantly alleviated the major issues of sampling error and inter-and intra-observer bias in conventional histological scoring systems. Furthermore its validated high sensibility to the fine changes of fibrosis pattern in cirrhosis samples, in combination with its algorithm of inherent structural interpretation, can be promising for substaging and characterizing the dynamic process of cirrhosis, with the particular etiological features being defined as well. This is undoubtedly desired in current clinical practice. The establishment of *q*Fibrosis could be exemplified as a successful and innovative integration of advances in medicine, biology and engineering. SQB assessment tool such as *q*Fibrosis is expected to facilitate improving the "gold standard" role of liver biopsy in hepatology medicine.[54]

Conclusion and Perspective

There are many clinical needs, and academic mysteries as well, related to hepatic fibrosis and cirrhosis wanting for us to understand. Given the introduction of the innovative SBQ assessment strategy, there are generally two directions in which the relevant research could move on forward. First is the clinical application in specifically stratified CLD conditions. For example, we need to further develop and validate our tools for defining the various surrogate markers for monitoring and predicting regression of hepatic fibrosis and cirrhosis; even further, we could replace the categorical labels with the corresponding continuous variables which obviously can more precisely and dynamically reflect the particular changes of histological fibrotic pattern in individual patients. Furthermore, there are some interesting areas in basic Hepatology we may explore. For example, it has long been observed in clinical medicine that advanced fibrosis and cirrhosis is prone to be oncogenic in CLD liver,[5] however, there still lacks direct and systematic *in situ* observation at tissue level, mainly due to the roughness of current definition of pathological conditions of fibrotic tissue. Keeping in mind that SBQ tool is able to provide the architectural information of fibrotic fibers with high resolution of up to cellular level; we may preset the variable panels of fine changes of hepatic fibrosis as a serial quantitative conditions to find out the critical tissue environmental parameters amiable for oncological genesis, development or metastasis. In conclusion, as histological examination will not be replaced of being one of the most important clinical references in a foreseeable future, by interfacing modern medicine with advanced bioengineering, innovative cutting edge technologies, such as SBQ, hold the great potential to facilitate the "good standard" liver biopsy assessment to accelerate the rapid evolution of clinical practices and studies in CLD.

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