



Novel assessment of hepatic iron distribution by synchrotron radiation X-ray fluorescence microscopy

Kinoshita, Hisoka

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Novel assessment of hepatic iron distribution by synchrotron
radiation X-ray fluorescence microscopy

走査型蛍光 X 線顕微鏡による肝組織内微量金属元素の新しい解析法
とその臨床病理学的意義

木下 秘我、堀 裕一、福本 巧、大東 琢治、篠原 邦夫、林 祥剛、具 英成

神戸大学大学院医学系研究科医科学専攻 肝胆膵外科学
(指導教員：具 英成教授)

木下 秘我

Key words: hepatitis C virus, iron, liver fibrosis, oxidative stress, synchrotron radiation
X-ray fluorescence microscopy, SRXRF

Novel assessment of hepatic iron distribution by synchrotron radiation

X-ray fluorescence microscopy

Hisoka Kinoshita • Yuichi Hori • Takumi Fukumoto • Takuji Ohigashi • Kunio

Shinohara • Yoshitake Hayashi • Yonson Ku

Correspondence to Y. Hori

Departments of Surgery, Division of Hepato-Biliary-Pancreatic Surgery, Kobe
University Graduate School of Medicine, Kobe 650-0017, Japan.

Tel.: +81-78-382-6302, Fax: +81-78-382-6307 e-mail: horiy@med.kobe-u.ac.jp.

H. Kinoshita • T. Fukumoto • Y. Ku

Departments of Surgery, Division of Hepato-Biliary-Pancreatic Surgery, Kobe
University Graduate School of Medicine, Japan

Y. Hayashi

Department of Pathology, Division of Molecular Medicine & Medical Genetics, Kobe
University Graduate School of Medicine, Japan

T. Ohigashi

Research Organization of Science & Engineering, Ritsumeikan University, Japan

K. Shinohara

Advanced Research Institute for Science and Engineering, Waseda University, Japan

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Abstract

Excess iron deposition in the liver is known to be hepatotoxic and may exacerbate liver injury. However, little is known about the iron distribution in the lobule due to the lack of a highly sensitive detection method. The aim of this study is to determine iron distribution in the lobule of human liver by means of synchrotron radiation X-ray fluorescence (SRXRF) microscopy. The mapping of the trace elements were done with use of SRXRF microscopy (SPring-8, Japan) and were compared with the results of staining by Berlin-blue and oxidative stress marker. Iron deposits were distributed predominantly in periportal hepatocytes in the normal liver. Its distribution indicated a decreasing gradient from the periportal area to the perivenous area. This distribution was consistent with the formation of the oxidative stress markers, suggesting that hepatocytes in the periportal area may be predominantly primed by iron-induced free radical damage even in the normal liver. On the other hand, iron deposits in the periportal area were more intense than those in the centrilobular area in both the liver with chronic hepatitis C and the cirrhotic liver. In conclusion, elemental mapping by the SRXRF microscopy was a highly sensitive method for the detection and mapping of elements such as iron and copper in liver sections.

Introduction

Iron is an essential metal for DNA synthesis, oxygen transport, and energy production; however, excess iron accumulation causes organ dysfunction through the production of reactive oxygen species (ROS).¹ Iron accumulation in the liver is common in patients with chronic liver diseases, especially in patients with chronic hepatitis C virus (HCV) infection.^{2,3} Excess iron deposition in the liver is hepatotoxic and may exacerbate liver injury.⁴ Nevertheless, little is known about the mechanism of iron accumulation in the liver. Impediments to such studies have been difficulties in measuring metal elements in tiny samples and in mapping localization of iron deposits on liver samples. For these purposes, conventional histochemical staining of tissue sections has been used to examine the local iron deposits. However, the sensitivity of the existing histochemical methods is not enough, and therefore they can detect only relatively highly concentrated hepatic iron deposits, thereby making a detailed relative distribution of the iron contents impossible.

Synchrotron radiation X-ray fluorescence (SRXRF) microscopy is a powerful non-destructive analytical technique that can be used to image the distribution of metal elements and provides complement for biochemical information.^{5,6} The purpose of this study was to use this method to obtain iron mappings of normal livers, chronic hepatitis

C (CHC) livers and the cirrhotic livers due to HCV, and to investigate the relationship between the distribution of iron accumulation and oxidative stress or fibrosis in the liver.

Materials and methods

Collection and preparation of tissue samples

Resected normal liver specimens were obtained from five living-donors, resected liver specimens from five patients with CHC, and resected liver specimens from five patients with liver cirrhosis due to HCV. Approval from the Kobe University Graduate School of Medicine ethical review committee was obtained. In addition, all patients in this study had signed a consent. Liver samples were fixed in 10% neutral buffered formalin and embedded in paraffin. 5 μ m sections of liver samples were used for H&E staining, Berlin blue staining, and immunohistochemistry. The sections for SRXRF microscopy were mounted on a polyimide foil Kapton (DuPont TORAY).

Synchrotron radiation X-ray fluorescence (SRXRF) microscopy

A schematic image of the SRXRF microscopy system is shown in Fig. 1. The optical

system was constructed at BL47XU of a synchrotron radiation facility, the SPring-8 (Harima, Japan). Intensity of monochromatic X-rays from the SPring-8 is extremely high so that much higher sensitivity can be achieved than by using a laboratory X-ray source. Furthermore, the intensity of X-rays is very stable at the top-up operation mode. Stability of the incident beam is very important for the SRXRF because quantitative measurements highly depend on it. In fact, fluctuation of the intensity of the incident beam was less than 1% during the measurement in each sample. These advantages of the synchrotron radiation facility are quite suitable for the SRXRF microscopy experiment.

Monochromatic X-rays at the energy of 13.0 keV was used as an incident beam. The incident beam was focused onto a sample by an X-ray focusing device, a Fresnel zone plate. The Fresnel zone plate was designed to be relative large diameter (774 μm) so that the incident beam was used quite efficiently. At focal point (i.e. at the sample) photon flux of $\sim 4 \times 10^{11}$ (photons / sec) was accumulated. From this photon flux, sensitivity of below 0.1 fg was estimated. In this system, the region of interest on the sample was precisely set to the focal spot of the X-ray microbeam by using an optical microscope. The focal spot size of the microbeam at the sample position was 8.5 (H) x 6.0 (V) μm^2 . The X-ray fluorescence from the sample was detected by an energy

dispersive detector, a silicon drift detector (SDD). In the energy profile by the SDD, we detected the specific peaks corresponding to the X-ray fluorescence of K α -lines from iron (6.40 keV), zinc (8.64 keV), and copper (8.05 keV). Two-dimensional elemental distributions of these elements were obtained by scanning the samples on the focal spot of the X-rays in horizontal and vertical direction point by point and mapping the intensities. The scan pitch was same as the focal spot size of the microbeam, 8.5 μm in horizontal direction and 6.0 μm in vertical direction with each dwell time of 1 sec per a point. Totally, it took about 7 hours to measure the area of 1.1 x 1.8 mm² on the sample. On the elemental distributions, red spots indicate high intensity deposits of each element, while blue spots mean low intensity deposits.

In principle, the intensity of the X-ray fluorescence is proportional to the quantity of the elements at the same focal spot and the intensity of the incident beam.⁷ Since the intensity of the incident beam of the SPring-8 is very stable, relative intensities were comparable as the difference in the relative contents in the same arrangements of detection system with the adjustment of the incident X-ray irradiation, dwell time and other physical parameters.

Immunohistochemistry

Immunohistochemistry was performed as described previously⁸ using mouse anti-KP1 antibody 1:100 (abcam), mouse anti-4-Hydroxy-2-nonenal (4-HNE) antibody 1:100 and mouse anti-8-Hydroxy-2'-deoxyguanosine (8-OHdG) antibody 1:100 (Japan Institute for the Control of Aging, Japan) and the Chemmate detection Kit (peroxidase/3,3'-diaminobenzadine, mouse or rabbit) (Dako).

Results

Distribution of iron deposits in the normal liver represents in a zone-dependent gradient

Conventional Berlin blue staining showed no iron deposits (Fig. 2d) in the normal lobule of the liver (Fig. 2a). This is consistent with previous papers. On the other hand, the elemental map by the SRXRF microscopy indicated that iron deposits were distributed predominantly in periportal hepatocytes (Fig. 2b). Moreover, its distribution indicated a decreasing gradient from the periportal area (zone 1) to the perivenous area (zone 3). High fold magnification images for Berlin blue stain and iron deposition by the SRXRF microscopy confirmed high sensitivity of SRXRF and a zone-dependent gradient of iron deposits, respectively (Fig. 3). Five normal liver specimen showed a similar results. In contrast, copper deposits were not detected in hepatocytes in the

normal liver (Fig. 2c). KP1 staining showed that Kupffer cells were scattered throughout the whole hepatic lobule (predominantly in the periportal area), thus indicating that iron deposits detected by the SRXRF microscopy do not show hemosiderin or iron incorporated by Kupffer cells (Fig. 2e). In addition, the formation of 4-Hydroxy-2-nonenal (4-HNE), a reliable marker of oxidative stress in the form of lipid peroxidation,⁹ and 8-Hydroxy-2'-deoxyguanosine (8-OHdG) was observed to increase predominantly in the periportal hepatocytes (Figs. 2f, 4). Omission of the primary antibodies showed a lack of immunostaining (data not shown).

Distribution of iron deposits in the liver from CHC

In the liver from the patients with CHC, H&E stain showed portal fibrosis with few septums (Fig. 5a). Berlin blue stain showed very few iron deposits around the portal fibrosis (Fig. 5d). In contrast, the elemental map by the SRXRF microscopy clearly demonstrated that iron deposits in the periportal area are more intense in comparison to those in the centrilobular area (Fig. 5b). KP1 staining showed Kupffer cells to be scattered throughout hepatic lobule, indicating that iron deposit detected by the SRXRF microscopy showed the accumulation both in hepatocytes and Kupffer cells (Fig. 5e). High fold magnification images for Berlin blue stain indicated the iron uptake by

Kupffer cells (Fig. 6). In contrast, no copper deposits were detected in the liver with CHC (Fig. 5c). The formation of 4-HNE was increased predominantly in the periportal hepatocytes (Fig. 5f).

Distribution of iron deposits in the cirrhotic liver with HCV

In the cirrhotic liver with HCV (Fig. 7a), Berlin blue stain showed only a few iron deposits on hepatocytes and Kupffer cells (Fig. 7d, e). In contrast, the elemental map by the SRXRF microscopy clearly demonstrated that iron deposits in the periportal area of each cirrhotic nodule are more intense in comparison to those in the centrilobular area (Fig. 7b), although each cirrhotic nodule showed different amounts of iron deposits. Moreover, copper deposits by the SRXRF microscopy showed a similar distribution of iron deposits (Fig. 7c). Notably, a fibrous septum around the cirrhotic nodule indicated few iron deposits and no copper deposits (Fig. 7b, 7c), while Kupffer cells, shown by KP1 staining, were accumulated on a fibrous septum (Fig. 7e). High fold magnification images for Berlin blue stain indicated not only the iron uptake by Kupffer cells, but deposits in the periportal hepatocytes (Fig. 8). The formation of 4-HNE was diffusely distributed throughout the cirrhotic nodules (Fig. 7f).

Discussion

Iron is absorbed from the diet through duodenal enterocytes. Dietary ferric iron (Fe^{3+}) is reduced by the ferric reductase to ferrous iron (Fe^{2+}). Ferric iron binds to transferrin and is carried to the liver in the blood. Excess iron can be harmful to the organism, in part through the generation of oxygen radicals.¹⁰ Excess iron deposits in the liver are hepatotoxic and may exacerbate liver injury through the generation of ROS that increases oxidative stress.^{4 11-13} While conventional Berlin blue staining represents the accumulation of Fe^{3+} and hemosiderin, Fe^{2+} deposits that induce oxidative stress, have not been measured due to the lack of a highly sensitive method. Magnetic resonance imaging (MRI) has been proposed as a sensitive and specific tool for assessing tissue iron overload.^{14, 15} While MRI exhibits an advantage in monitoring a ubiquitous iron accumulation in the tissue, SRXRF microscopy has an advantage over MRI because it yields an elemental map of tissue microstructures.⁵ Previous reports indicated that the precision of X-ray fluorescence spectrometry (XRF) analysis was of the same order of magnitude as for atomic absorption spectrometry with respect to measurement of iron, copper, and zinc.¹⁶ Moreover, there was good agreement between results obtained by XRF in human liver biopsy specimens and large samples.¹⁷ The SRXRF microscopy has a potential for higher sensitivity and better spatial resolution to detect elements than

conventional XRF due to much higher intensity of X-ray source, synchrotron radiation, so that the analysis of the spatial distribution of elements in tissue sections became possible at high resolution. Although we need calibration of the system to obtain the absolute quantity of the elements, the intensity of the X-ray fluorescence is proportional to the quantity of the elements at the same focus size and intensity of the incident beam.⁷ The present study revealed iron distribution in the liver by the SRXRF microscopy and clarified iron metabolism in the normal, chronic hepatitis C, and cirrhotic liver. Iron forms, including free iron, stored iron or heme-iron were not distinguished by the SRXRF microscopy, suggesting that the current method detected all forms of iron. Further detailed studies should be required to compare among different disease stages.

The normal liver showed that iron deposits were predominantly in the periportal hepatocytes and decreased in a zone-dependent manner. This is the first time iron distribution has been detected in the normal human liver. In contrast, no zinc and copper deposits were detected in the same specimen (Fig. 2c, data not shown), thus suggesting that iron deposits by the SRXRF microscopy were both sensitive and specific. Of note, the iron distribution was consistent with the formation of the oxidative stress marker, 4-HNE and 8-OHdG proteins. From the viewpoint of oxidative stress,

hepatocytes in the periportal area may be predominantly primed by iron-induced free radical damage even in the normal liver. In addition, iron deposits in the periportal hepatocytes can favor HCV replication and infection, although it is difficult to examine a liver specimen during the early stage of HCV infection.

Both in chronic hepatitis C and in liver cirrhosis, iron deposits were predominant and more intense in the periphery of the nodule in comparison to the centrilobular area. HCV is thought to produce oxidative stress through multiple mechanisms that include chronic inflammation, iron overload, and liver injury.¹⁸ Previous studies reported a positive correlation between serum iron index and alanine aminotransferase in patients with chronic hepatitis C, thus suggesting that the excess iron could be related to its release from destroyed hepatocytes as a result of liver injury by HCV infection.^{19, 20} Another possible explanation is that HCV might affect the expression of proteins important in modifying iron trafficking during inflammation/host response, such as hemochromatosis gene (HFE), ferritin, ferroportin, or hepcidin.²¹⁻²³ Of note, the present study was also the first to clarify the distribution of copper deposits in the cirrhotic liver, but not in the normal liver and the liver with CHC. Previous studies reported that the copper level was also noted in sera of patients with hepatitis and increased copper levels might result from inflammatory responses.^{24, 25} The significance

of the copper distribution in the cirrhotic liver remains to be studied further.

Another important point to note is the mechanism underlying iron overload and liver fibrosis. Dietary iron overload in rats induced iron deposition predominantly in the periportal hepatocytes, with very little in the reticuloendothelial cells, a distribution pattern close to that observed in patients with hereditary hemochromatosis.²⁶ Portal areas are enlarged with increased collagenous tissue. This portal fibrous tissue extends between the periportal hepatocytes at sites of maximal iron deposition.^{26,27} This phenomenon is very similar to the current result. Furthermore, few iron deposits were observed within the fibrous septum in spite of the accumulation of Kupffer cells.

Therefore, the periportal sites of maximal iron deposition, but not the centrilobular sites, are initially induced to liver fibrosis. In general, both activated Kupffer cells and activated hepatic stellate cells (HSC) are thought to be key players in liver fibrosis.^{12,28} However, little attention has been given to the point that liver fibrosis occurs initially in the periportal area, although Kupffer cells and HSCs exist in hepatic sinusoid and the space of Disse, respectively, throughout the liver. The iron deposits in the peripheral area of the cirrhotic nodule, but not in the fibrous septum, suggest the following mechanism. HCV infection induces iron deposits predominantly in the periportal hepatocytes. Iron overload in hepatocytes causes oxidative stress. Oxidative

stress or oxidative stress-induced cytokines can activate Kupffer cells and HSCs and induce collagen synthesis. Consequently, iron deposits persist in the periphery of the cirrhotic nodule and disappear in the completed fibrous septum. The KP1-positive cells in the fibrous septum may be macrophages that migrate from the blood. Since the expression of HCV core protein or HCV infection has not been detected by immunohistochemistry until now, further investigation is needed to clarify the mechanism underlying the association between iron distribution and HCV infection.

Since the complex interplay between HCV infection, iron homeostasis and inflammation in the development of liver disorders, including fibrosis, cirrhosis, and carcinogenesis is unequivocal, the elemental map provided by the SRXRF microscopy might potentially be a new strategy to establish an effective and innovative therapy for liver disease.

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Figure legends

Fig. 1. The schema of synchrotron radiation X-ray fluorescence (SRXRF) spectroscopy on the liver specimens.

Fig. 2. Iron deposits in the normal liver were predominantly detected in the periportal hepatocytes. A normal lobule stained with (A) H&E (B) iron deposits by SRXRF microscopy, (C) copper deposits by SRXRF microscopy, (D) Berlin blue stain (E) KP1 and (F) 4-HNE immunostaining. Original magnification was 40X. Scale bar = 200 μm .

Fig. 3. High fold magnification images for Berlin blue stain and iron deposition by SRXRF confirmed high sensitivity of SRXRF and a zone-dependent gradient of iron deposits in the normal liver, respectively. Scale bar = 50 μm .

Fig. 4. Oxidative stress proteins in the normal liver. A normal lobule stained with (A) 4-HNE and (B) 8-OHdG immunostaining. Original magnification was 100X. Scale bar = 100 μm .

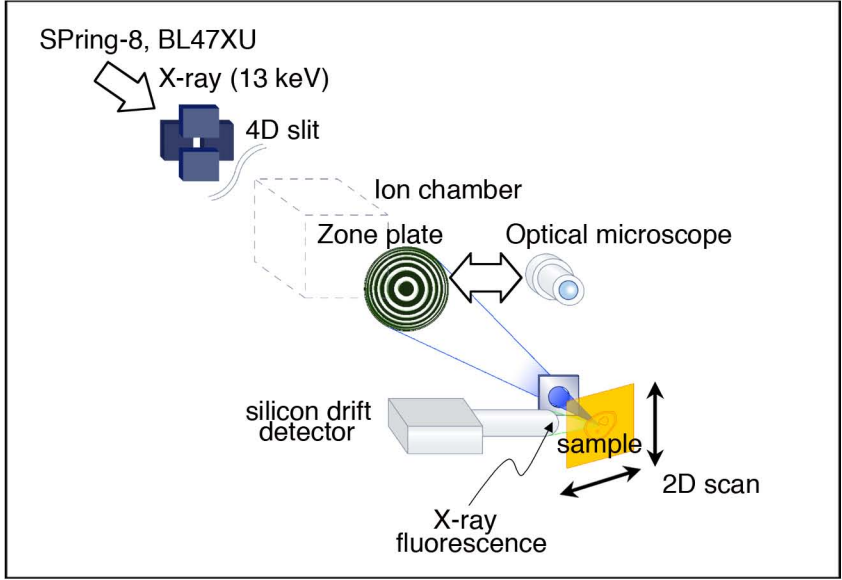
Fig. 5. Iron deposits in a liver with chronic hepatitis C (Stage 5) were also predominantly detected in the periportal area. Images are stained with (A) H&E, (B) iron deposits by SRXRF microscopy, (C) copper deposits by SRXRF microscopy, (D) Berlin blue stain, (E) KP1 and (F) 4-HNE immunostaining. Original magnification was 40X. Scale bar = 200 μm .

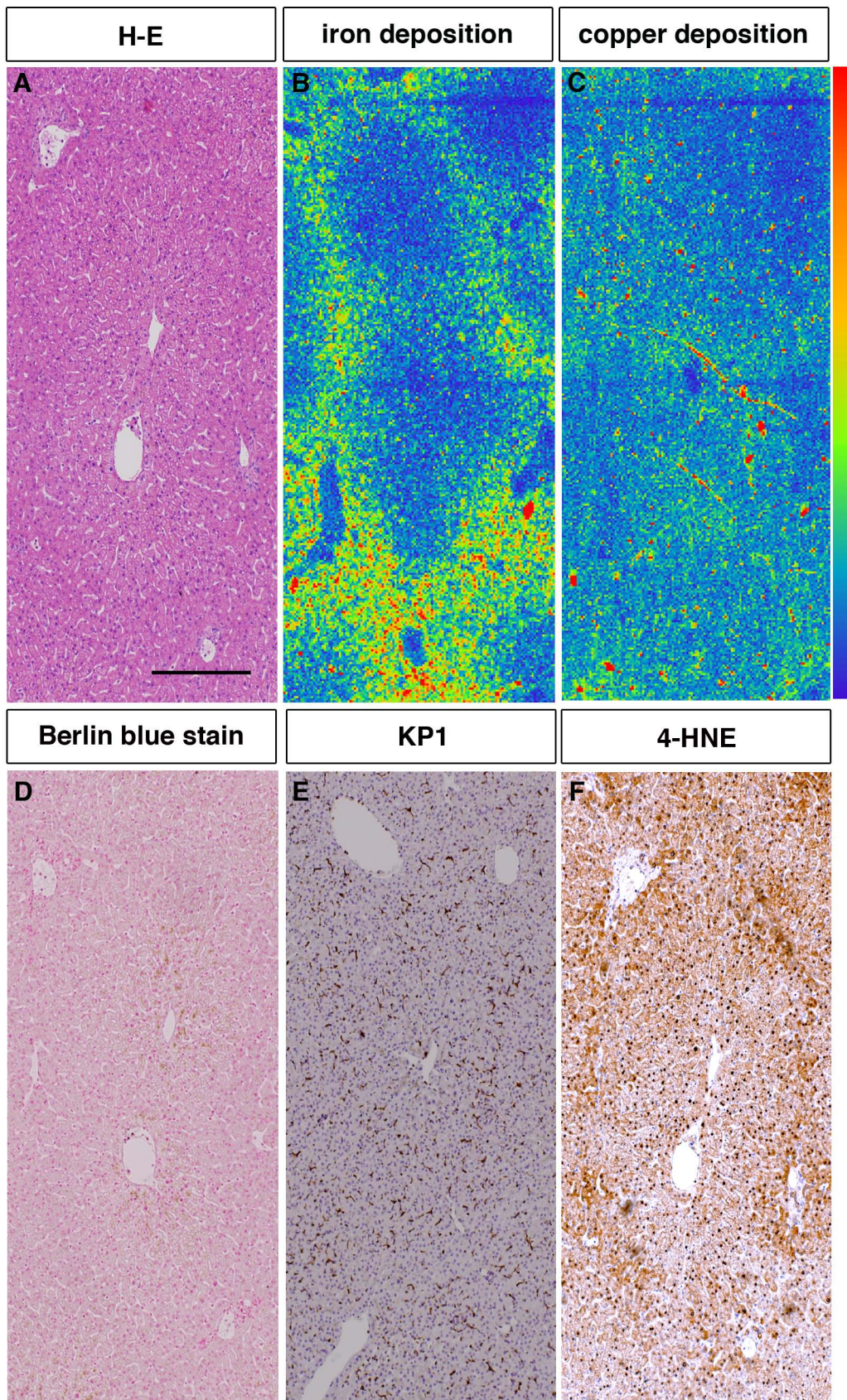
Fig. 6. High fold magnification images for Berlin blue stain in the liver with chronic

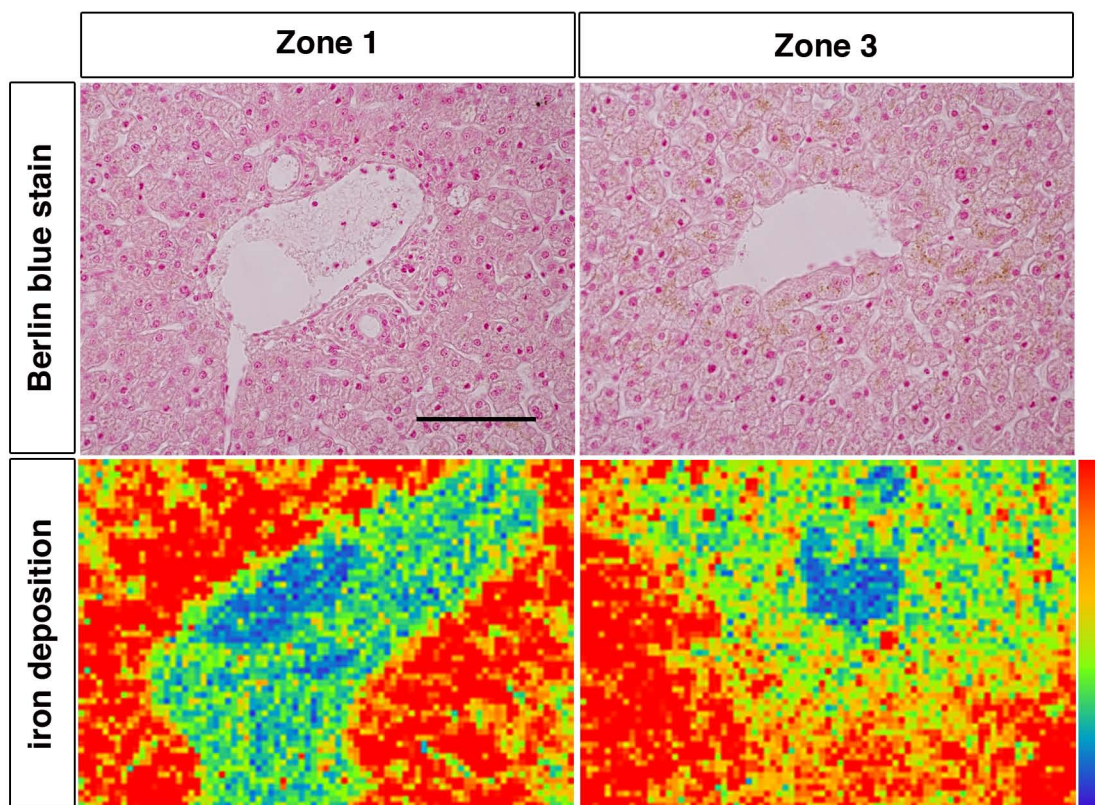
hepatitis C. Scale bar = 50 μm .

Fig. 7. Iron deposits in the cirrhotic nodules were predominantly detected in the peripheral area. The cirrhotic nodules are stained with (A) H&E, (B) iron deposits by SRXRF microscopy, (C) copper deposits by SRXRF microscopy, (D) Berlin blue stain, (E) KP1 and (F) 4-HNE immunostaining. Original magnification was 40X. Scale bar = 200 μm .

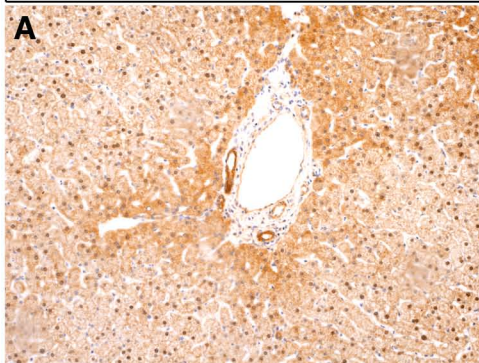
Fig. 8. High fold magnification images for Berlin blue stain and iron deposition by SRXRF confirmed high sensitivity of SRXRF and a distribution of iron deposits in the cirrhotic liver, respectively. Scale bar = 50 μm .







4-HNE



8-OHdG

