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RESEARCH

Nucleometric Study of Anisonucleosis, Diabetes and Oxidative Damage in Liver Biopsies of Orthotopic Liver Transplant Recipients with Chronic Hepatitis C Virus Infection

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Abstract Anisonucleosis is defined as a morphological manifestation of nuclear injury characterized by variation in the size of the cell nuclei. It has been described in variety of benign conditions and is most pronounced in dysplasia and malignancy. To better understand the pathogenesis of anisonucleosis in liver diseases, this study focused on hepatocyte anisonucleosis in biopsies of liver transplant recipients who developed recurrent chronic hepatitis C virus (HCV) infection. Post transplant surveillance liver biopsy specimens were evaluated employing light microscopy, immunohistochemistry, digital image analysis, and nucleometry for histopathological analyses, measurement of nuclear size, and quantification of tissue expression of oxidative marker 8-hydroxy-2′deoxyguanosine (8-OHdG). Our aim in this study was to determine whether there were any independent associations between hepatocyte anisonucleosis and various clinicopathological parameters. These features included patient age, body mass index, gender, race, donor age, live versus cadaveric donor status, history of diabetes mellitus, history of tacrolimus and cyclosporine therapy, duration post transplant and parameters of hepatitis activity index, fibrosis index, steatosis, and oxidative tissue damage in formalin fixed paraffin embedded (FFPE) liver biopsies as determined by immunohistochemistry using 8-OHdG, an indicator of hydroxyl radical mediated tissue damage. Our findings suggested that in liver transplant recipients with recurrent chronic HCV infection, hepatocyte anisonucleosis is more pronounced in individuals with diabetes mellitus (p=0.0016), and among those who have heightened hepatic expression of the oxidative damage marker 8-OHdG (p=0.0053). Further studies are necessary to determine whether anisonucleosis is an independent marker for diabetes or oxidative damage.

Keywords Diabetes · Hepatitis C virus (HCV) · Hepatocyte anisonucleosis · Image analysis · Nucleometry · Oxidative damage · 8-hydroxy-2′deoxyguanosine (8-OHdG) immunomarker

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Normal hepatocytes exhibit minimal variation in overall size but their nuclei vary in size, number, and ploidy [1]. Anisonucleosis is a pattern of hepatocyte injury that is best defined as the variation in the size of cell nuclei [2]. Hepatocyte anisonucleosis is noted in various human conditions. It is described to increase with age, [1] present in a variety of reactive conditions, and is most pronounced in both hepatocellular dysplasia and carcinoma [2–5]. Reports of anisonucleosis associated with non-neoplastic liver diseases include drug induced liver disease [6] and

cirrhosis [3]. In animal studies, anisonucleosis has been described in experimentally induced amebic abscess [7] in gerbils and as a morphologic change that occurs early during the course of diethylamine-induced hepatic oncogenesis in mice [8]. The pathogenesis of anisonucleosis in non-neoplastic liver diseases in humans is not well understood; however, it is likely that anisonucleosis in these conditions represent the morphologic manifestation of nuclear injury by a variety of mechanisms.

We have observed hepatocyte anisonucleosis in benign hepatic conditions including chronic hepatitis C virus (HCV) infection, metabolic and toxic steatohepatitis, drug induced hepatitis, autoimmune hepatitis and other chronic liver diseases in humans (unreported observations). Chronic HCV infection is a significant cause of morbidity and mortality [9]. How viral and non-viral factors including diabetes interact during chronic HCV infection to cause liver injury and in some cases, hepatocellular carcinoma, is not completely clear [10, 11]. However, oxidative damage is thought to play an important role. Standard immunohistochemical methods utilizing 8-hydroxy-2′deoxyguanosine (8-OHdG [Genox, Baltimore, MD]), an indicator of hydroxyl radical mediated damage to cellular nucleic acids including nuclear and mitochondrial DNA [12] for measurement of oxidative damage, can be employed on formalin fixed paraffin embedded liver tissues (FFPE) [13, 14]. In a previous study, we identified a positive relationship among diabetes, oxidative liver damage, and the progression of viral hepatitis in chronic HCV re-infected post-transplant patients [14]. Recurrence of chronic HCV infection following transplantation is universally guaranteed [15].

To better understand the pathogenesis of anisonucleosis in chronic HCV infection, we chose to initially focus our efforts on liver transplant recipients with chronic HCV re-infection because this group provides a relatively uniform extent of liver involvement and fibrosis at baseline, immediately following transplantation. A series of consecutive surveillance post transplant liver biopsies obtained at appropriate clinical time intervals were evaluated to allow a retrospective assessment of progression of fibrosis over time. Our goal was to determine whether any association existed among hepatocyte anisonucleosis and various clinical parameters, namely patient age, body mass index (BMI), gender, race, donor age, live versus cadaveric donor status, history of diabetes mellitus, history of tacrolimus and cyclosporine therapy, duration post transplant and parameters of hepatitis activity index, fibrosis index, steatosis, and oxidative tissue damage in FFPE liver biopsies as determined by immunohistochemistry using 8-hydroxy-2′deoxyguanosine (8-OHdG), an indicator of hydroxyl radical mediated tissue damage [12, 14] in liver transplant recipients with chronic HCV infection.

In the current study, we found a significant association among hepatocyte anisonucleosis, history of diabetes mellitus and enhanced immunostaining for the oxidative damage marker 8-OHdG in biopsies of liver transplant recipients with chronic hepatitis C virus infection.

Materials and Methods

Study Population

This study was performed on 33 liver biopsies derived from 19 orthotopic liver transplant (OLT) recipients who developed chronic HCV re-infection as detected by HCV RNA polymerase chain reaction and had compatible biopsy findings over a 20 month period. Protocol liver biopsies were performed at 6 and 12 months and annually after transplantation. The subjects had an average of 2 and at least 1 to 3 biopsies. Normal control tissues ($n=3$) were obtained from otherwise normal liver margins of resection specimens for benign hepatic conditions.

The Institutional Review Board at the University of Illinois at Chicago has approved this study protocol.

Liver Histology

Hematoxylin and eosin and trichrome stained liver biopsies were evaluated by a liver pathologist who did not have preview to clinical outcomes. Sampling adequacy of the biopsies was determined and only those that met criteria or containing 10 or more portal triads [16] were included in the study. Features of viral necroinflammatory activity, fibrosis stage, steatosis, rejection, and other significant findings were assessed based on published standard guidelines [16–19]. Biopsies that manifested any significant fatty liver disease with more than mild steatosis [19] were excluded from the study. Likewise, biopsies that demonstrated features of cellular rejection, [18] dysplasia [20, 21] or malignancy were withdrawn.

Nucleometry to Determine Average Hepatocyte Nuclear Size and Calculations for the Range of the Average Hepatocyte Nuclear Size as an Expression of Anisonucleosis

Hematoxylin stained 4 μm thick sections of FFPE liver tissues were scanned at 200× magnification and the average hepatocyte nuclear size was determined using the ImageScope® System (Aperio Technologies, Inc, Vista, CA). Ten random regions within each biopsy with an average of 160 (103–282) hepatocyte nuclei per region, or a total of at least 1,600 (1,126–2,147) hepatocyte nuclei per biopsy were analyzed. Anisonucleosis is determined by obtaining the range or the difference between the highest and lowest value of the average nuclear size among ten

Table 1 Patient demographics and clinical information

Patient demographics and clinical information (n=19)	
Characteristics	Data
Patient age (median years; range)	50 (17–69)
Gender, (female/male)	8/11
Race (African American, Caucasian, Hispanic, other)	5/4/8/2
Patient BMI (median; range)	26 (19–59)
Living donor transplant (yes/no)	6/13
Pretransplant diabetes mellitus (yes/no)	6/13
Posttransplant diabetes mellitus (yes/no)	9/10
Immunotherapy (tacrolimus/cyclosporine)	13/6
Donor age (median years; range)	35 (18–64)
Duration posttransplant (median years; range)	1.53 (0.52–7.19)

regions per biopsy. The median was calculated. The range of the average hepatocyte nuclear size, as an expression of anisonucleosis, was categorized as low if less than or equal (≤), or high if greater (>) than, the median.

Immunohistochemistry and Image Analysis for Detection and Assessment of Oxidative Damage

Serial 4 μm slices were obtained from paraffin embedded tissue specimens. Sections were transferred to glass slides and dried overnight at 60°C. Following deparaffinization and dehydration through graded alcohols and xylene, a 35-min antigen retrieval was performed in a pressure cooker. The slides were placed in a coplin jar containing antigen retrieval solution (10× high pH antigen retrieval solution, Dakocytomation, Carpinteria, CA). Hydrogen peroxide was applied, followed by the addition of mouse anti-8-OHdG (Genox, Baltimore, MD) at a dilution of 1:300 for 30 min at room temperature. Immunohistochemical staining was performed using the Envision + System-HRP (DAB) (DAKO Cytomation, Carpinteria, CA) method. A negative control for each case was generated through substitution of subclass matched monoclonal antibody against an irrelevant antigen. A slide showing liver with chronic HCV cirrhosis served as a positive control for each case.

The percentage of immunoreactive hepatocytes displaying nuclear positivity for the 8-OHdG was evaluated by digital image analysis using liver tissues obtained from the uninvolved margins of human liver resection specimen for benign liver conditions (negative control, n=3), and a total of 33 biopsies derived from 19 OLT recipients with chronic HCV infection. Ten random regions with an average of 160 (103–282) hepatocytes per region or a total of at least 1,600 (1126–2147) hepatocytes per biopsy were analyzed. A 4 tier staining intensity score (scale 0–3) corresponding to absent, low, medium, and high staining intensity was provided. To standardize the variability in staining intensity in a region, a staining index, representing the sum of the product of staining intensity and percentage of hepatocyte nuclei exhibiting that degree of intensity, was established and calculated based on published criteria [22–24]. For example, staining index = staining intensity of (3 X 0.4 [corresponding to 40% of positively staining hepatocyte nuclei in that region]) + (2 X 0.3) + (1 X 0.2) + (0 X 0.1) = 2.0. The range of the hepatocyte staining indices within the 10 regions was identified in each biopsy sample and the median was determined. The range of hepatocyte staining index was categorized as low if ≤, or high if > than the median.

Calculation of Fibrosis Index as a Measure of Progression of Fibrosis over Time

The stage of fibrosis was estimated by trichrome stain using published parameters [17]. Fibrosis index, representing the stage of fibrosis (F0-4) divided by the duration post transplant in years was calculated for each of the 33 samples [14, 25].

Clinical Data

Clinical data and demographic features were collected retrospectively from patients' electronic medical records. The use of oral antidiabetic or insulin treatment was the basis for inclusion for a diagnosis of diabetes mellitus. Categorical variables were provided for diabetes status, gender, use of live versus cadaveric donor, and use of immunosuppressant tacrolimus versus cyclosporine. Data were grouped into high or low scores using the median as the cut-off value for patient age, donor age, body mass index, and duration in days post transplant. Patients were categorized as African American, Caucasian, Hispanic or other ethnicity.

Table 2 Comparison of nuclear size and range of average hepatocyte nuclear size as an expression of anisonucleosis between normal liver control tissues and liver biopsies with chronic hepatitis C virus obtained from OLT recipients

	Normal liver control (n=3)	Liver biopsies of hepatic transplant recipients with chronic HCV infection (n=33)
Average hepatocyte nuclear size (μm²)	37.86–49.60	32.30–65.75
Range of average hepatocyte nuclear size or anisonucleosis (μm²)	3.69–4.39	3.41–21.20

Table 3 Comparison of staining index and range of staining index for 8-OHdG between normal liver control and liver biopsies of orthotopic liver transplant (OLT) recipients with chronic hepatitis C virus infection

	Normal liver biopsy control tissues (n=3)	Liver biopsies of hepatic transplant recipients with chronic HCV infection (n=33)
Staining index	0.02–0.40	0–2.20
Range of staining index	0.12–0.19	0.03–1.43

Statistical Analysis

Data analysis was performed using two tailed Fischer exact test and Chi square test with Yates correction to account for small sample size.

Results

Study Population

The median patient age for the study population at the time of biopsy was 50 (17–69) years; 42% (8/19) were female; 26% (5/19) were African American, 21% (4/19) Caucasian, 42% (8/19) Hispanic, and 11% (2/19) of other ethnicity. The median body mass index (BMI) was 26 (19–59), duration post transplant in years was 1.53 (0.52–7.19), 32% (6/19) had pre-transplant, and 47% (9/19) had post transplant diabetes mellitus. Sixty eight percent (13/19) used tacrolimus and 32% (6/19) cyclosporine immunotherapy. The median liver donor age was 35 (17–64) years, 32% (6/19) had a live, and 68% (13/19) had a cadaveric donor (Table 1).

Hepatocyte Nucleometry

Average, Range of Average, and Median Range of Average Hepatocyte Nuclear Size in Normal Liver Control Tissues (n= 3) The average hepatocyte nuclear size in the normal liver control tissues was 42.98 μm^2 (37.86 to 49.60). In normal liver control tissues the range of the average hepatocyte nuclear size was 3.69 to 4.39 μm^2 with a median range of 4.21 μm^2.

Average, Range of Average, and Median Range of Average Hepatocyte Nuclear Size as an Expression of Anisonucleosis in Liver Biopsies of Orthotopic Liver Transplant Recipients with Chronic HCV Infection (n= 33) The average hepatocyte nuclear size in liver biopsies of OLT recipients with HCV infection ranged from 32.30 to 65.75 μm^2, with a range of average hepatocyte nuclear size or anisonucleosis of 3.41 to 21.20 μm^2, and a median range of 6.17 μm^2.

Categorical Designation of Hepatocyte Anisonucleosis in the Liver Biopsies of OLT Recipients with Chronic HCV Infection (n= 33) Hepatocyte anisonucleosis </= to the median range (6.17 μm^2) was categorized as low, or designated as high if > the median range (6.17 μm^2). Fifty two percent (17/33) of the biopsies had a low, and 48% (16/33) had a high hepatocyte anisonucleosis. Table 2 illustrates the average nuclear size and the range of the average nuclear sizes as an expression of anisonucleosis in normal liver control tissues and biopsies of orthotopic liver transplant recipients with chronic HCV infection.

Immunohistochemistry for Hepatocyte Oxidative Damage Marker

Staining Index Range, and Median Range of Staining Index for Oxidative Damage Marker (8-OHdG) in Normal Liver Control Tissues (n= 3) The range of staining index for 8-OHdG in normal liver control tissues was 0.12 to 0.19 with a median of 0.13.

Staining Index Range, and Median Range of Staining Index for Oxidative Damage Marker (8-OHdG) in Liver Biopsies of Orthotopic Hepatic Transplant Recipients with Chronic HCV Infection (n= 33) The range of staining index for 8-OHdG among the subjects was 0.03 to 1.43 with a median range of 0.24.

Categorical Designation into Low or High Range of Staining Index for 8-OHdG A staining index score of </= to the median, 0.24, was categorized as low, and high if >0.24. Fifty two percent (17/33) of the biopsies had a low, and 48% (16/33) had a high 8-OHdG staining index score. Table 3 shows the staining index and range of staining

Table 4 Summary of values for stage of fibrosis, duration post transplant in years and fibrosis index of liver transplant recipients with chronic hepatitis C virus infection (n=33)

Components of fibrosis index	Data (n=33)
Stage of fibrosis (median / range)	2 (0–4)
Duration post-transplant (years) (median / range)	1.53 (0.52–7.19)
Fibrosis index (FI) (median / range)	0.86 (0–3.85)
Low FI (≤0.86) (percentage / [low FI / total])	52 (17/33)
High FI (>0.86) (percentage / [high FI / total])	48 (16/33)

Table 5 Association among hepatocyte anisonucleosis, oxidative damage marker and clinical data

Clinical & pathological features	Number of biopsies	Range of the average nuclear size[a]		p value[b]
		High $> 6.1732\mu m^2$	Low $\leq 6.1732\mu m^2$	
DM[c] pre[d]: Yes	33	8	3	0.0707
DM pre: No	33	8	14	
DM post[e]: Yes	33	12	3	0.0016
DM post: No	33	4	14	
DM pre or post: Yes	33	12	3	0.0016
DM pre or post: No	33	4	14	
Patient Age ≤ 50	33	11	11	0.805
Patient Age > 50	33	5	6	
Donor Age ≤ 35	29	10	6	0.139
Donor Age > 35	29	4	9	
Live Donor	33	7	4	0.281
Cadaveric Donor	33	9	13	
Female	33	6	7	0.829
Male	33	10	10	
Hispanic	33	6	7	0.829
African American, Caucasian, other	33	10	10	
African American (AA)	33	7	3	0.141
Hispanic, Caucasian, other	33	9	14	
Caucasian	33	1	5	0.174
AA, Hispanic, other	33	15	12	
BMI[f] ≤ 26	33	9	9	0.849
BMI > 26	33	7	8	
8-OHdG index Range: low (≤ 23.84)	33	4	13	0.0053
8-OHdG index Range high (> 23.84)	33	12	4	
8-OHdG index Average: low(≤ 40.91)	33	11	6	0.084
8-OHdG index Average: high (> 40.91)	33	5	11	
Duration post transplant: ≤ 557 d	29	11	6	0.139
Duration post transplant: > 557 d	29	4	8	
Tacrolimus	33	11	15	0.224
Cyclosporin	33	5	2	
Fibrosis 0–2	33	13	13	0.737
Fibrosis > 2	33	3	4	
Fibrosis index ≤ 0.86	33	7	10	0.494
Fibrosis index > 0.86	33	9	7	
Portal inflammation: 0–2	33	15	17	0.484
Portal inflammation: > 2	33	1	0	
Piecemeal necrosis: 0–2	33	14	16	0.601
Piecemeal necrosis: > 2	33	2	1	
Lobular inflammation: 0–2	33	15	13	0.656
Lobular inflammation: > 2	33	2	3	
Steatosis: 0–2	33	16	17	1
Steatosis: > 2	33	0	0	

[a] This value was calculated by finding the average nuclear size from 10 fields with an average of 160 hepatocytes per field or a total of at least 1600 hepatocytes per biopsy. The range of the average nuclear size per biopsy was then calculated by taking the difference between the highest and lowest average nuclear size among the ten regions. The median value for the range of the average nuclear size, as an expression of anisonucleosis, for all 33 biopsies was then calculated and that number is 6.1732 μm^2

[b] $p<0.05$: significant

[c] Type II diabetes mellitus

[d] Diagnosed before transplantation

[e] Diagnosed after transplantation

[f] BMI (body mass index)

index in normal liver control and liver biopsies of transplant recipients with chronic hepatitis C virus infection.

Fibrosis Index and Categorical Designation into Low or High Fibrosis Index (n=33)

The stage of fibrosis of the 33 biopsies ranged from 0 to 4, with a median fibrosis stage of 2. The duration post transplant in years of the 33 biopsies ranged from 0.52 to 7.19. The fibrosis index, or the stage of fibrosis (scale 0–4) / duration in years post transplant, was determined for all 33 samples. The range of the fibrosis indices was 0 to 3.85, with a median of 0.86 and 75th percentile value of 1.33.

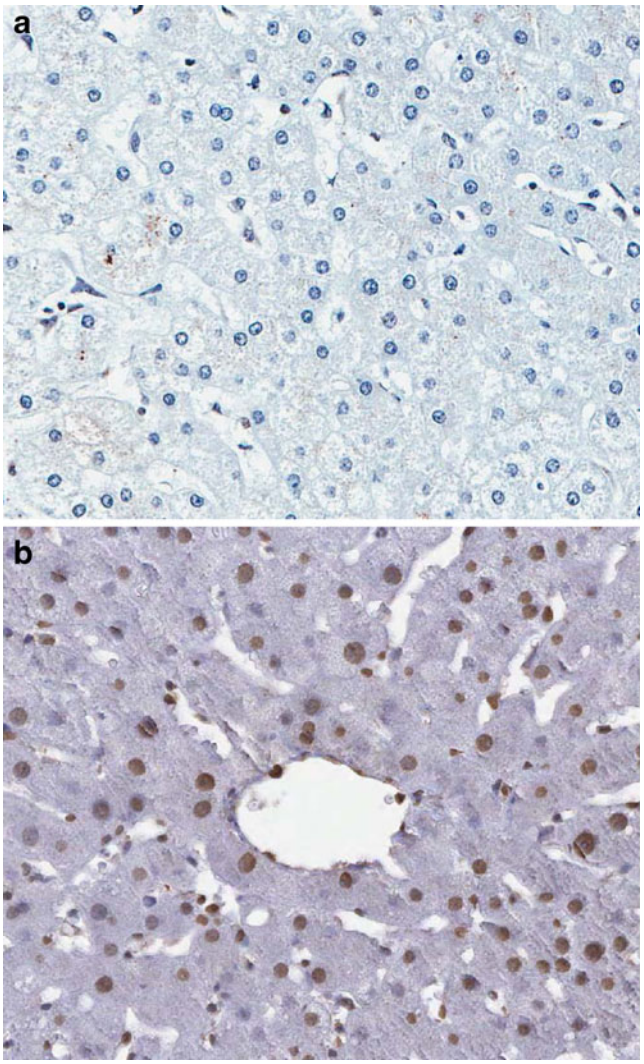

Fig. 1 Liver tissues reactive with the oxidative damage marker 8-OHdG by immunohistochemistry. **a** Normal liver tissue demonstrating no evidence of significant 8-OHdG immunoreactivity and low variation of nuclear size; **b** liver tissue derived from an orthotopic liver transplant patient with chronic hepatitis C virus infection demonstrating 8-OHdG immunoreactivity in more than 60% of hepatocytes and high variation of nuclear size (magnification = 200×)

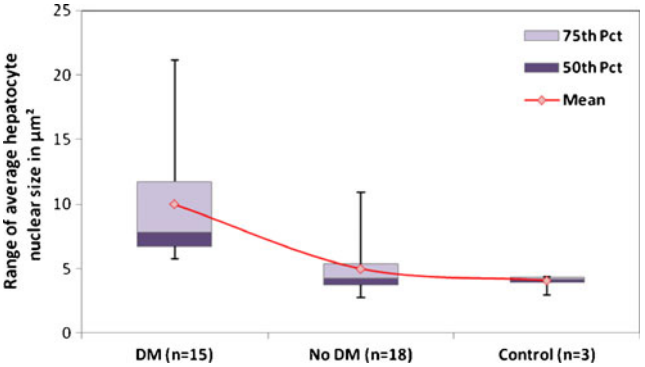

Fig. 2 Box and whisker plot comparing range of average hepatocyte nuclear size as an expression of anisonucleosis in liver biopsies of transplant recipients with chronic HCV infection with and without diabetes mellitus (n=33) and in normal liver control tissues (control n=3) (p=0.0016)

The fibrosis index was categorically designated low if the value is </= to the median (0.86), or high if > the median. Fifty two percent (17/33) of the biopsies had a low and 48 % (16/33) had a high fibrosis index score. Table 4 shows a summary of these findings.

Associations Among Anisonucleosis and Various Clinical Parameters

In the current study, we did not find any significant associations among anisonucleosis and patient gender (p=0.83), patient age (p=0.81), race (p=0.14 to p=0.83), donor age (p=0.14), patient BMI (p=0.85), use of live versus cadaveric donor liver (p=0.28), duration in years post transplant (p=0.14) and use of immunosuppressant tacrolimus versus cyclosporine (p= 0.22). There were no significant associations among anisonucleosis, fibrosis index (p=0.49) and the parameters of hepatitis activity index including portal inflammation (p=0.48), peri-

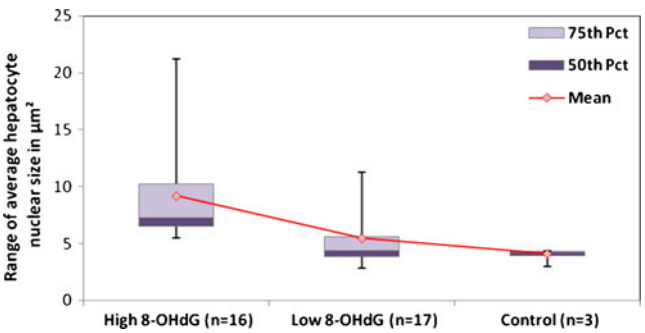

Fig. 3 Box and whisker plot comparing range of average hepatocyte nuclear size as an expression of anisonucleosis in cases with high or low range of staining indices for the oxidative damage marker, O-8HdG, in liver biopsies of transplant recipients with chronic HCV infection (n=33) and in normal liver control tissues (n=3) (p=0.0053)

Table 6 Summary of histopathological findings including degree of steatosis, fibrosis index, and hepatitis activity index in liver biopsies of orthotopic liver transplant recipients with chronic HCV infection

Summary of histopathological findings (n=33)	
Features	Median (range)
Steatosis	0 (0–2)
Fibrosis index	0.86 (0–3.85)
Parameters of hepatitis activity index [17]	
Portal inflammation	2 (0–3)
Periportal inflammation	2 (1–3)
Lobular inflammation	2 (1–3)
Fibrosis	2 (0–4)

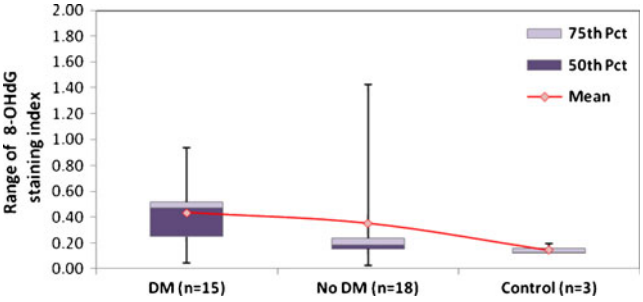

Fig. 4 Box and whisker plot showing the positive association of range of 8-OHdG staining index and history of diabetes mellitus in liver biopsies of transplant recipients with chronic HCV infection (n=33) (p=0.0015)

portal inflammation (p=0.60), lobular inflammation (p=0.66), and stage of fibrosis (p=0.74) (Tables 5 and 6).

Association Between Anisonucleosis and Oxidative Damage and Diabetes Mellitus

There was a significant association among anisonucleosis, enhanced immunohistochemical expression of tissue oxidative damage marker 8-OHdG (p=0.0053), and history of post-transplant diabetes mellitus (p=0.0016) in liver transplant recipients with chronic HCV infection (Table 5, Figs. 1, 2 and 3).

Association Between Range of Staining Index and History of Diabetes Mellitus

There was a significant association between increased range of immunohistochemical expression of the oxidative damage marker 8OHdG and history of diabetes mellitus (p=0.0015) (Table 7, Fig. 4).

Discussion

The current study addresses the association of hepatocyte anisonucleosis, diabetes, and immunoexpression of tissue oxidative damage marker in post transplant chronic hepatitis C virus re-infected patients. Here, two new observations were noted. Hepatocyte anisonucleosis was more common in cases with diabetes mellitus, and anisonucleosis was associated with amplified expression of an immunomarker of oxidative damage in liver transplant recipients with chronic HCV infection. In contrast, the current study found no significant associations among hepatocyte anisonucleosis and patient gender, patient age, race, BMI, donor age, use of live versus cadaveric donor, duration post transplant, use of immunosuppressant tacrolimus versus cyclosporine, components of the hepatitis activity index, namely portal, periportal, and lobular inflammation, fibrosis, and fibrosis index.

Although this study focused on anisonucleosis in post transplant chronic hepatitis C virus infection, it is worthwhile to compare the characteristics of anisonucleosis within this group to the other entities in which it has been described. Anisonucleosis in benign, non-neoplastic, and neoplastic conditions could appear morphologically similar, but their pathogenesis is likely to be at least partially different. In humans, anisonucleosis along with cholestasis, was observed in drug induced liver injury following the use of anti-inflammatory medication (clinoril) for treatment of rheumatological conditions [6]. We have observed anisonucleosis in both metabolic and toxic steatohepatitis, drug induced hepatitis, autoimmune hepatitis and other chronic liver diseases (unreported observations). Hepatocyte anisonucleosis, bi-nucleation, intranuclear inclusions were frequently observed and marked in fine needle aspiration biopsies of livers with abscess, hepatitis, cirrhosis, focal nodular hyperplasia and primary liver carcinomas [26].

Table 7 Association of range of 8-OHdG staining index with history of post transplant diabetes mellitus in liver biopsies from orthotopic liver transplant (OLT) recipients with chronic hepatitis C virus infection (p=0.0015)

Range of 8-OHdG staining index (median=0.24) Median (range)	DM (n=15) 0.48 (0.05–0.93)	No DM (n=18) 0.19 (0.03–1.43)	Total n=33 0.24 (0.03–1.43)	p value
High range of 8-0HdG (> 0.24)	12	4	16	0.0015
Low range of 8-0HdG (</= 0.24)	3	14	17	
Total	15	18	33	

However, nuclear morphometry detected differences between hyperchromasia, nuclear enlargement and anisonucleosis characterizing non-cancerous liver lesions, and hepatocellular carcinoma [5]. While pronounced anisonucleosis and hyperchromasia were similarly identified in malignant and non-malignant portions of cirrhotic livers with hepatocellular carcinoma, DNA ploidy correlated with the mean nuclear area in malignant but not in non-malignant hepatocytes [4]. A karyometric analysis comparing liver cell dysplasia and hepatocellular carcinoma contested the precancerous nature of large cell dysplasia. Anisonucleosis lacked nuclear indentations and contained iron deposits in foci of large cell dysplasia, while these features were not observed in hepatocellular carcinoma lesions [2]. Anisonucleosis is a morphologic change that occurs early during the course of diethylamine-induced hepatic oncogenesis in mice [8]. In humans, there is insufficient data to support any link between anisonucleosis as an early cell injury response in chronic liver disease and malignant transformation of hepatocytes.

Our current findings suggest that anisonucleosis represents a morphologic manifestation of nuclear injury caused by oxidative stress and diabetes during chronic HCV infection. The mechanisms by which oxidative damage and diabetes affect nuclear size of hepatocytes are not clear from our study. The protein NS5A and the core protein of hepatitis C virus have been shown to cause oxidative stress in cell culture systems by modifying intercellular calcium flow and thereby increasing levels of mitochondrial reactive oxygen species (ROS) production [27, 28]. Oxidative damage of nuclear macromolecules, including DNA and proteins, may lead to morphological changes itself or may induce reactive processes that affect nuclear size. Elevated levels of ROS increase insulin resistance [29]. Previously we demonstrated an association among fibrosis progression, oxidative damage and diabetes in liver transplant recipients with chronic HCV infection [14]. Oxidative damage can induce hepatocyte fibrosis through hepatic stellate cell activation, transforming growth factor-beta (TGF-ß) and collagen synthesis leading to fibrosis [30]. Hyperinsulinemia develops in advanced fibrosis or cirrhosis of any cause due to peripheral insulin resistance [31]. The stromal cells in fibrosis have been shown to be a source of growth factors such as platelet derived growth factor (PDGF), fibrosis growth factor (FGF), vascular endothelial growth factor (VEGF), and hepatocyte growth factor (HGF) in chronic liver disease [32]. It is possible that upregulation of these growth factors in advanced fibrosis or in diabetes participate in the pathogenesis of anisonucleosis by providing stimuli for nuclear enlargement in hepatocytes. It is likely that the ill effects of oxidative damage are most significant in advanced fibrosis.

Although the molecular mechanisms involved are not revealed by our study, observations reported here suggest for the first time that oxidative damage and diabetes affect nuclear size of hepatocytes in post transplant HCV re-infection. The strengths of this study include a well characterized group of patients with a defined follow-up period, and elimination of bias by both utilizing a blinded study design and nucleometry. Confines of the present study include a relatively limited sample size and restriction to post-transplant HCV infected subjects. Nonetheless, these initial findings give us novel insight into the pathogenesis of anisonucleosis in liver transplant recipients with chronic hepatitis C virus infection. To expand these observations, we have initiated further studies addressing any possible association of fibrosis progression, diabetes, oxidative damage and anisonucleosis in other benign liver conditions including metabolic and toxic steatohepatitis, drug induced liver injury, autoimmune hepatitis, and other viral hepatitides.

In conclusion, the current study provides initial evidence to suggest that there might be an association among anisonucleosis, oxidative damage, and diabetes mellitus in orthotopic liver transplant recipients with chronic hepatitis C virus infection. Further studies with a larger patient cohort will be necessary to determine whether anisonucleosis is a definitive marker of oxidative stress or diabetes in chronic hepatitis C virus infection.

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