DNA ploidy and liver cell dysplasia in liver biopsies from patients with liver cirrhosis

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There is controversy among pathologists when assessing the presence or absence of liver cell dysplasia in liver biopsies taken from cirrhotic patients. The objective of the present study was to determine the DNA ploidy pattern of hepatocytes of patients with liver cirrhosis and its relationship to liver cell dysplasia. A total of 48 male patients diagnosed with liver cirrhosis based on clinical, laboratory and histopathological criteria were included in the study. A liver biopsy was taken from each patient; one part of the biopsy was subjected to histopathology, and the other to flow cytometry. The histopathological examination revealed liver cell dysplasia in 60% of patients with liver cirrhosis (62% of them had large cell dysplasia [LCD] and 38% had small cell dysplasia [SCD]). Abnormal DNA content (aneuploidy) was found in 81.5% of positive liver cell dysplasia specimens and found only in 11.1% of negative liver cell dysplasia specimens, with a statistically significant difference (P<0.001). Aneuploidy was found more commonly in LCD but without significant difference (P>0.05) in comparison with SCD. In conclusion, SCD (similar to LCD) is also associated with aneuploidy and elevated DNA index, and may carry the same risk for progression to hepatocellular carcinoma.

Key words: *Flow cytometry; Liver cell dysplasia; Liver cirrhosis; Ploidy*

L'ADN-ploïdie et la dysplasie hépatocytaire dans des biopsies hépatiques de patients cirrhotiques

Il existe une controverse entre pathologistes à l'égard de l'évaluation de la présence ou de l'absence de dysplasie hépatocytaire dans les biopsies hépatiques prélevées sur des patients cirrhotiques. La présente étude vise à déterminer le profil d'ADN-ploïdie des hépatocytes des patients cirrhotiques et le lien de ce profil avec la dysplasie hépatocytaire. Au total, 48 patients cirrhotiques de sexe masculin diagnostiqués d'après des critères cliniques, de laboratoire et histopathologiques ont participé à l'étude. Une biopsie hépatique a été prélevée sur chacun d'eux. Une partie de la biopsie a été soumise à une histopathologie et l'autre, à une cytométrie de flux. L'examen histopathologique a révélé une dysplasie hépatocytaire dans 60 % des cas de cirrhose (62 % constituant des cas de dysplasie à grandes cellules [DGC] et 38 %, de dysplasie à petites cellules [DPC]). Un contenu d'ADN anormal (aneuploïdie) a été observé chez 81,5 % des spécimens positifs de dysplasie hépatocytaire, mais seulement chez 11,1 % des spécimens négatifs, la différence étant statistiquement significative (P<0,001). L'aneuploïdie était plus souvent décelée dans les cas de DGC, mais la différence n'était pas significative (P>0,05) par rapport à ceux de DPC. Pour conclure, la DPC (similaire à la DGC) s'associe aussi à l'aneuploïdie et à un index d'ADN élevé, et elle peut comporter le même risque de progression vers un carcinome hépatocellulaire.

Cirrhosis of the liver, the end-stage of chronic liver disease of different etiology, shows a worldwide distribution affecting all races and ages of both sexes (1,2). The structural changes to the liver as a result of cirrhosis determine two essential features: the reduction in hepatocellular function and the development of portal hypertension. The intensity of each of these disorders ranges from very mild, with no clinical manifestation, to severe, leading to death (3). Pathologists do not disagree about characteristics of liver cell dysplasia, but they agree about its nature (ie, regenerative, degenerative or precancerous changes). Liver cell dysplasia was first reported in some patients with liver cirrhosis (4). It is considered to be a premalignant condition (5,6). On the other hand, reports by Omata et al (7) and Nakashima et al (8) have not supported the premalignant nature of liver cell dysplasia. In an attempt to search for some parameters that would make it possible to predict a potentially high risk of evolution from liver cirrhosis to hepatocellular carcinoma (HCC), the DNA content of hepatocytes in patients with liver cirrhosis with or without development of HCC has been investigated using morphometric analysis of nuclear size and of DNA content and ploidy pattern by flow cytometry (FCM) (9-11). FCM has also been applied to the analysis of the nuclear DNA content of adenomatous hyperplasia occurring in cirrhotic livers by Hoso and Nakanuma (12). The aim of the current study was to determine the DNA ploidy pattern in hepatocytes of patients with liver cirrhosis and its relationship to the liver cell dysplasia.

PATIENTS AND METHODS

Patients

The present study included 48 patients with liver cirrhosis (all men, aged 40 to 55 years) who presented to the outpatient clinic, Internal Medicine Department, Mansoura University Hospitals, Mansoura, Egypt. The diagnosis of cirrhosis was based on clinical

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laboratory and histopathological criteria. Percutaneous liver biopsy was performed in each patient using a Trucut needle (unicut-biopsy-needle; 1,6 mmx, 115 mm long, 16 gauge) to obtain liver tissue for histopathological examination and FCM analysis of hepatocyte DNA ploidy status. Liver specimens from six patients subjected to cholecystectomy and with normal liver based on histopathological examination were used as controls. Schistosomiasis and viral hepatitis B and C were the main etiological agents for liver disease in these patients. The clinical severity of cirrhosis was assessed using the Child-Pugh classification (13). Most patients were classified as Child's class A and B. Laboratory investigations included complete blood count, serum albumin, alanine aminotransferase (ALT), serum bilirubin and prothrombin time. Patients with marked or tense ascites, hepatic focal lesion (eg, tumours) detected by abdominal sonography and impaired coagulation (prothrombin activity 50% or less and low platelet count 50×10^9/L or less) were excluded from the study.

Histological investigation

Histological analysis was made on formalin fixed paraffin embedded sections. Five millimetre thick liver sections, prepared and stained with hematoxylin and eosin, were analyzed for the presence of large cell dysplasia (LCD) according to Anthony et al (4) and small cell dysplasia (SCD) according to Watanabe et al (14). The activity of liver cirrhosis was scored using the histological activity index of Ishak et al (15).

FCM

FCM analysis was performed with a coulter EPICS profile II flow cytometer (Coulter Corp, USA), configured with a 488 nm argon ion laser as reported in our previous study (16). Briefly, single cell suspensions were prepared by mechanical dissociation of fresh biopsy specimens in RPMI-1640 medium (Sigma Chemical Co, USA) followed by filtration through a piece of fine nylon mesh (45 µm pore size) and centrifugation to remove debris and cell clumps. Cells were permeabilized with triton X-100 (Sigma) followed by staining with propidium iodide (Sigma) as a DNA-specific fluorochrome. Peripheral blood lymphocytes were used as an external standard for tissue material. A total of 20,000 events per sample were acquired. DNA aneuploidy was defined as any population with a distinct additional peak(s) or the presence of a tetraploid population greater than 15%. DNA histograms were analyzed using a cytological software (Coulter Corp). An histogram showing only one G0/G1 peak was considered as diploid cells (normal) and that showing two distinct peaks was considered as aneuploid cells. DNA index was measured by determination of the ratio of DNA content of the aneuploid peak to the DNA content of the diploid peak. A DNA aneuploid population has a DNA index higher than 1.1 N in 10% or more of the nuclei. The relative amounts of cells in S and G2+M phases of the cell cycle were determined using the computer program "Cytology" (Coulter Corp) and weres used as measures for cellular proliferative activity.

Statistical analyses

All parameters were transferred to an IBM (Yorktown Heights, USA) PC/AT compatible computer for analysis using the statistical analysis program package Instat Software for Science, version 2.3 (Graphpad Software Inc, USA). Data were characterized by their means and standard deviation (mean ± SD). Fisher's exact test was used to compare ploidy among patients with and without dysplasia. A one-sided unpaired *t* test was used to compare between the proliferation activity (S+G2M) in normal and cirrhotic livers. $P<0.05$ was considered to be significant.

TABLE 1
Histopathological findings of liver cirrhosis

Histopathological findings	n	%
Type of liver cirrhosis		
Macronodular	47	97.9
Micronodular	1	2.1
Activity of cirrhosis		
Active	21	43.75
Inactive	27	56.25
Liver cell dysplasia		
Without dysplasia	19	39.6
Small cell dysplasia	11	22.9
Large cell dysplasia	18	37.5

RESULTS

Histopathological data

All 48 percutaneous needle liver biopsy specimens were valid for histopathological evaluation. Macronodular cirrhosis was present in 97.9% of the patients, while only one patient (2.1%) had micronodular cirrhosis. Active cirrhosis was found in about 44% of the patients, as suggested by the presence of piecemeal necrosis and bridging or multilobular hepatic necrosis with lymphocytic infiltration. Inactive cirrhosis was found in about 56% of the patients, with clean-cut borders between nodules and septa with inflammatory mononuclear cells within the fibrous septa but not extending into the parenchyma (Table 1). Liver cell dysplasia was found in 60% (29 of 48) of patients with cirrhosis. SCD was found in 38% (11 of 29) of the patients, and LCD was found in 62% (18 of 29) (Figure 1).

DNA FCM analyses

All (100%) six normal liver specimens showed diploid histogram with only one G0/G1 peak; the DNA index (DI) was one and aneuploid cells were not found in any of the normal liver specimens (Table 2). FCM evaluation was done for only 45 liver biopsies. Three liver biopsies were excluded due to an inadequate number of cells to be analyzed. Twenty-four (53.3%) biopsies were diploid, while 21 (46.7%) were aneuploid ($1.8<DI>1.1$). Tetraploidy ($2.5<DI>1.8$) was not found in any of the biopsies. The DI ranged from 1.0 to 1.32 (1.09±0.1) (Figure 2). The proliferative fractions (per cent of cells in S and G2/M phases of the cell cycle) in the normal liver group were 18.8% and in liver cirrhosis biopsies it was 26.85% showed a significant ($P<0.05$) increase. The cellular DNA content was not related to the activity of cirrhosis. Patients with liver cell dysplasia had a significantly ($P<0.008$) lower serum albumin level (35.7±5.3 g/L) than those without dysplasia (40.1±4.3 g/L), but there was no significant difference in serum albumin between cases with SCD or LCD. No significant correlation was found between bilirubin, ALT or prothrombin time, and LC0-D. Patients with liver cell dysplasia (SCD and LCD) had significantly ($P<0.0001$) higher DIs (1.13±0.09 and 1.18±0.16; respectively) than those without dysplasia (1.02±0.06). Abnormal DNA content (aneuploidy) was found in 81.5% of positive liver cell dysplasia specimens and found only in 11.1% of negative liver cell dysplasia specimens with a statistically significant difference ($P<0.001$).

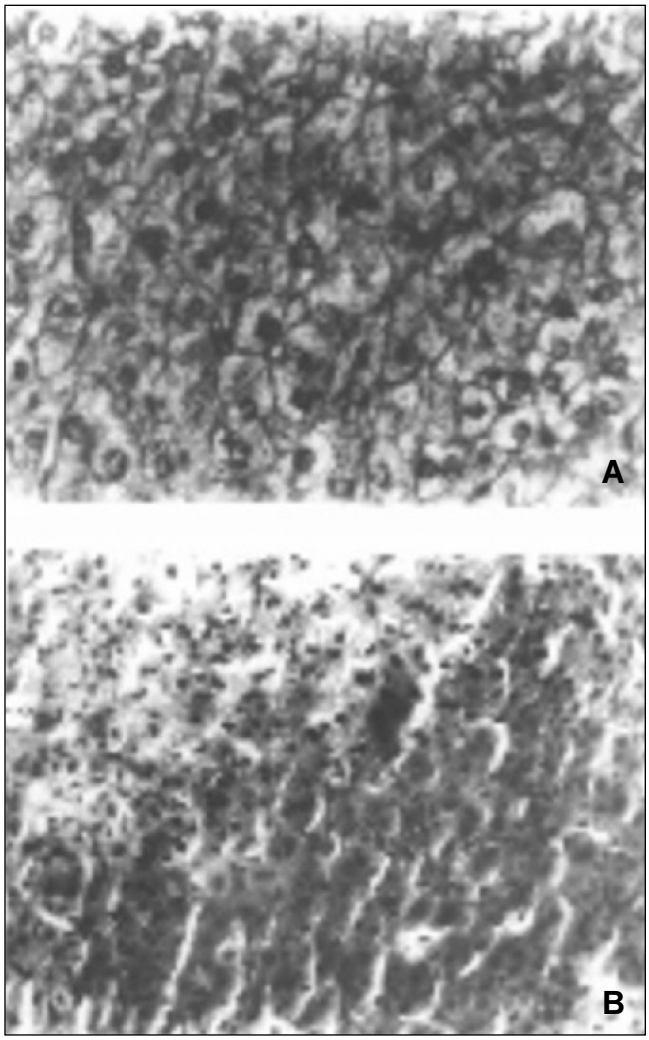

Figure 1) *Liver cirrhosis with liver cell dysplasia.* **A** *Large cell dysplasia. Scattered hepatocytes contain enlarged, darkly stained and vesicular, often pleomorphic, nuclei with irregularly clumped chromatin, conspicuous nucleoli and accentuated nuclear membranes.* **B** *Small cell dysplasia. Thick trabecula formed of multiple layers of hepatocytes with pleomorphic hyperchromatic nuclei with occasional vesicular and binucleated forms*

TABLE 2
DNA flow cytometric data for normal liver and cirrhotic liver without dysplasia, with large cell dysplasia and with small cell dysplasia

Characteristics	Normal liver	Liver cell dysplasia (n = 45)*		
		Without	SCD	LCD
DNA index				
Number	6	18	11	16
Mean	1	1.02	1.13	1.18
P†			<0.0001	<0.0001
Proliferative index of diploid				
Number	6/6	16/18	3/11	2/16
Mean % (S + G2M)	18.8	26	20	36.5
P†			>0.05	>0.05
Aneuploidy				
Number	0	2/18	8/11	14/16
Cases with aneuploidy (%)	0	11.1	72.7	87.5
P†			<0.002	<0.001
Aneuploid cells (%)				
Mean %	0	6.26	44.2	44.5
P†			<0.0001	<0.0001

**Three out of 48 liver biopsies were excluded due to inadequate number of cells to be analyzed using flow cytometry; †In comparison with cirrhotic patients without liver cell dysplasia. LCD Large cell dysplasia; SCD Small cell dysplasia*

Furthermore, aneuploidy was found more commonly in LCD compared to SCD, without significant difference (87.5% versus 72.7%, P>0.05) (Table 2). The percentage of aneuploid cells ranged between 4.90 to 73.95% (43.19±15.3%). Patients with liver cell dysplasia (SCD and LCD) had significantly (P<0.0001) higher percentage of aneuploid cells (44.2±8.4% and 44.5±14%; respectively) than those without dysplasia (6.26±18.45%). Active cirrhosis was significantly (P<0.05) associated with a higher proliferation index. There was a significant (P<0.05) inverse correlation between serum albumin levels and the percentage of aneuploid cells in liver tissues.

DISCUSSION

There is controversy among pathologists when assessing the presence or absence of liver cell dysplasia in liver biopsies taken from cirrhotic patients. FCM is a rapid technique, and can assess thousands of cells in a few minutes. Moreover, FCM can identify cases with high cell proliferation (high S phase and G2/M), which are very susceptible to different mutagenic agents or chemicals, which result in neoplastic transformation. FCM can therefore be considered as a better ancillary technique in the assessment of liver cell dysplasia because it is objective and not liable to misinterpretation or inter-observer discordance. In the present study, liver cell dysplasia was found in 60% of patients with liver cirrhosis; SCD in 38% and LCD in 62%. Our results are in agreement with the findings of Roncalli et al (17) and Lin et al (18). To determine the true nature of these cases with liver cell dysplasia, we used FCM to determine the DNA ploidy, cellular DNA content and proliferation index (PI). We found that 81.5% of cases that were histopathologically shown to have liver cell dysplasia also had aneuploidy. In addition, aneuploidy was found in 11.1% of biopsies without liver cell dysplasia. This may be due to the higher sensitivity of FCM. These results confirm the aneuploid nature of liver cell dysplasia (19,20). The DNA content of liver cells expressed as DI was significantly higher (P<0.0001) in cases with liver cell dysplasia than cases without liver cell dysplasia, in accordance with the findings of other investigators (17,21). Lee et al (22) proposed that LCD derives from derangements in the hepatocyte's normal process of polyploidization. Such derangements, possibly caused by chronic inflammation-induced DNA damage, could yield a population of enlarged liver cells with nuclear atypia and pleomorphism, frequent binuclearity and minimal proliferation. Accordingly, LCD would be a habitual feature of cirrhosis and a regular accompaniment of HCC, but would not represent a direct malignant precursor. Libbrecht et al (23) indicated that the presence of LCD in a needle liver biopsy of patients with viral-induced chronic liver disease is an independent risk factor for the development of HCC. In another study, foci of LCD showed no correlation with putative progenitor cells (24). One-half of the oncocytic and glycogen-storing foci contain 'progenitor cells', while more than one-half of the foci of SCD

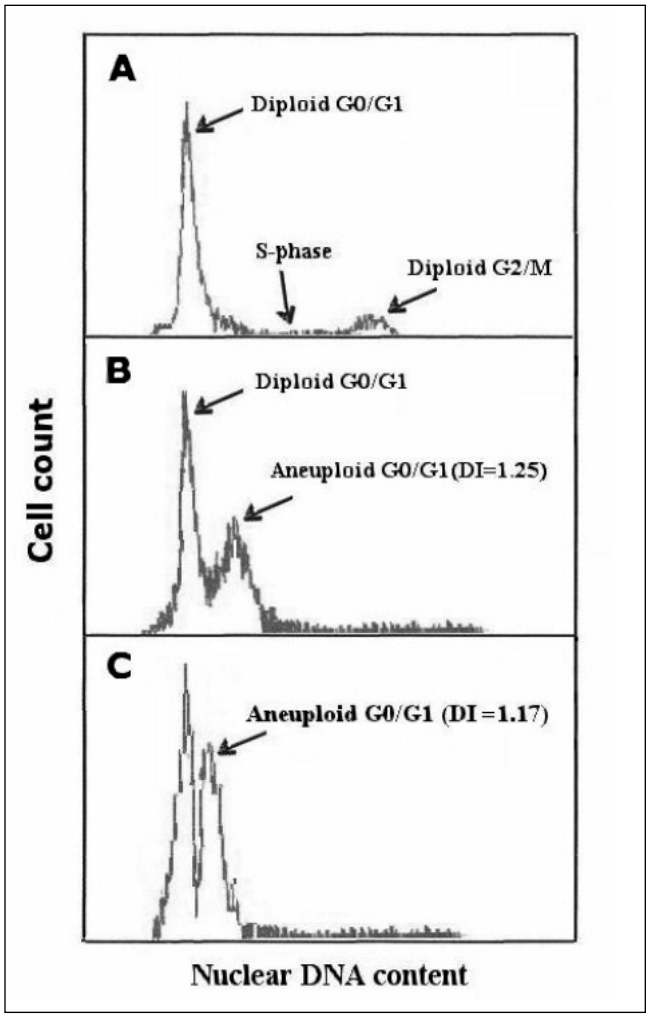

Figure 2) *Flow cytometric DNA analysis of* (**A**) *normal liver tissue (Diploid, DNA index [DI]=1),* (**B**) *liver cirrhosis tissue positive for large liver cell dysplasia and* (**C**) *liver cirrhosis tissue positive for small liver cell dysplasia (Aneuploid, DI= 1.17)*

consist of small cells with the same immunohistochemical phenotype as putative progenitor cells and intermediate hepatocyte-like cells, suggesting that differentiating putative progenitor cells can give rise to foci of SCD (24). At least two possibilities can be considered to explain the diploidy in liver cirrhosis biopsies with dysplasia. One is that there may be balanced translocations chromosomal rearrangements without change in chromosomal volume, point mutations, or deletions that cannot be identified by FCM measurement (25,26). The other possibility may due to the degree of quantitative change of chromosomes. It is difficult to identify a small change, such as a defect in the short arm of a chromosome or an abnormal or small sized chromosome. Furthermore, the aneuploidy was found more commonly in LCD compared to SCD, but no multiple aneuploid peaks (polyploidy) were found in either LCD or SCD. However, this point would need further study. Yet, we found that the cellular DNA contents showed insignificant relation with activity of cirrhosis, which is in agreement with Lin et al (18). Moreover, active cirrhosis was significantly ($P<0.05$) associated with a higher PI (S+G2M phases) than diploid cases with inactive cirrhosis and may due to liver response to cell injury (27). The value of detecting these cellular DNA changes in liver cirrhosis by FCM is of prognostic value in regards to early detection of preneoplastic changes in liver cirrhosis, since patients with high cell proliferation in cirrhotic livers were found to be at increased risk of developing HCC (28-30). Proliferating cell nuclear antigen and KI-67 by immunohistochemistry were useful for proliferative activity assessment of hepatocyte (31). Recently, Liu et al (32) suggested proliferating cell nuclear antigen, KI-67 and FCM analysis of S-phase fraction may be useful predictive markers of biological aggressiveness in laryngeal carcinoma. The presence of an aneuploid DNA pattern had been reported by Hoso and Nakanuma (12) to be a precancerous change in cases with atypical adenomatous hyperplasia of the liver. Yet, the presence of liver cell dysplasia, although not specific as a premalignant condition, could be considered as a serious landmark in cases of liver cirrhosis. In the present study, DNA aneuploidy was identified in 46.7% of patients with liver cirrhosis. However, Thomas et al (9) reported a lower incidence (25%) of DNA aneuploidy in patients with liver cirrhosis. This difference may be due to the use of archival material of paraffin-embedded blocks. In the current study, serum albumin levels was significantly lower ($P<0.008$) in patients with liver cell dysplasia than in patients without dysplasia. Moreover, a significant negative correlation ($P<0.05$) was found between the level of serum albumin and the percentage of aneuploid cells. This may be attributed to poor synthesis of albumin by these dysplastic hepatic cells as a result of change in the nuclear DNA contents of these aneuploid cells. The patients with active cirrhosis showed significantly higher ($P<0.05$) levels of both serum bilirubin and ALT. This significant difference may be due to the presence of necroinflammatory changes resulting from parenchyma injury. In our study, 44% of patients had active cirrhosis and inactive cirrhosis was found in 56% of patients with clean-cut borders between nodules and septa with inflammatory mononuclear cells within the fibrous septa but not extending to parenchyma (33). Further study will be performed on cases with aneuploid liver cirrhosis for close follow-up to detect early neoplastic changes to hepatocellular carcinoma. In conclusion, SCD (similar to LCD) is also associated with aneuploidy, elevated DI and may carry the same risk for progression to HCC.

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