Micronuclei and Broken Eggs in Human Liver Carcinogenesis

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Abstract. *Background: Micronuclei (MNi) and broken eggs (BE) are both considered sensitive markers of genotoxic damage and chromosomal instability. In humans, a high frequency of MNi is reported in both cirrhosis and hepatocellular carcinoma, but no information is available on MNi/BE expression in dysplastic nodules. MNi/BE formation may result in activation of the p53-mediated cell cycle checkpoint. Materials and Methods: MNi, BE (Feulgen staining) and immunohistochemical expression of p53 and Mib1 were assessed in 95 liver lesions representing the whole spectrum of liver carcinogenesis. Seven normal liver tissue samples served as controls. MNi and BE were assessed by video-assisted microscopy and expressed as a crude number per 1,000 hepatocytes. Results: MNi and BE were significantly more frequent in all the pathological samples than in the controls ($p<0.001$). A progressively increasing number of MNi/BEs was documented from cirrhotic nodules (CN) to large regenerative nodules (LRN), to dysplastic nodules (DN) and hepatocellular carcinoma (HCC) (test for trend; $p<0.001$). MNi were significantly more frequent in DN than in CN or LRN ($p=0.011$; $p=0.020$, respectively). Proliferative activity (Mib1) and p53 expression were significantly associated with MNi presence ($p<0.001$ and $p=0.031$, respectively). Conclusion: Chromosomal instability significantly increases throughout the multistep course of hepatocarcinogenesis. The similar prevalence of MNi and BEs in DN and HCC supports their strict biological similarity. A high prevalence of MNi/BE may identify a subset of (genetically unstable) cancer-prone cirrhosis cases.*

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Liver cancer is the final event in a cascade of genetic changes having their phenotypic counterpart in a spectrum of morphological alterations including: cirrhotic nodules (CN); large regenerative nodules (LRN); low-grade dysplastic nodules (LG-DN); high-grade dysplastic nodules (HG-DN) and full-blown hepatocellular carcinoma (HCC) (1-4).

The molecular mechanisms involved in virus-related liver carcinogenesis have yet to be completely elucidated, but increased cell turnover and direct viral damage both have a major role (5, 6). Chromosome instability, including structural chromosome anomalies and allele loss or gain, has been demonstrated in HCC (7, 8) and found to be closely associated with hepatitis B virus (HBV) infection (9, 10).

Micronuclei (MNi) are chromosomes or chromosome fragments left out of the daughter nuclei during nuclear division (11, 12). Chromosome breakage and mitotic apparatus dysfunctions are involved in the morphogenesis of MNi, which are generally considered a phenotypic expression of chromosome instability (13). Environmental clastogenic and aneuploidogenic (chemical and biological) agents are involved in MNi generation. Micronucleus assay (on lymphocytes and exfoliated cells) has been used as a multiple-end-point test of genomic instability, genotoxic exposure and early biological effects in human biomonitoring studies (12, 14, 15). MNi have been demonstrated in all stages of liver carcinogenesis in experimental models (*in vitro* and in animals) (16-18).

The so-called broken eggs (BE) are small amounts of genetic material attached to the main cell nucleus by a "Feulgen-positive filament". Like MNi, BE are considered indicators of genotoxic exposure resulting in chromosome aberrations (19).

TP53 is a cell gatekeeper gene inducing cell cycle arrest (or apoptosis) in genetically modified cells. MNi may activate the p53-mediated cell cycle checkpoint (20). The immunohistochemical assessment of both p53-protein and cell proliferation (Ki67 antigen [Mib1] expression) may provide information on the functional status of the system involved in preserving genetically stable cell populations.

In the only available study on humans, De Almeida *et al.* (21) found MNi in regenerative, macroregenerative and neoplastic liver nodules from cryptogenic and hepatitis C virus (HCV)-related cirrhosis, but the study notably did not include dysplastic nodules (the true pre-cancerous liver lesions).

The present study was designed to: assess MNi and BE over the whole spectrum of phenotypic lesions involved in human liver cell oncogenesis; to verify whether MNi/BEs occur to the same extent in HBV- and HCV-related lesions and to investigate any relationship between p53 expression, hepatocyte proliferation and MNi/BE formation.

Materials and Methods

Materials. Liver tissue samples were obtained from 45 consecutive patients (M/F 26/19; mean age 57.29±6.25 years, range 33-67, median 59), who underwent orthotopic liver transplantation (OLT) for HBV- and/or HCV-related cirrhosis (HBV=12, HCV=28, HBV/HCV=5). The HCV genotype was known in 17 cases (10 type 1b; 7 type 2). Patients given pre-OLT therapy to downstage HCC were excluded. The explanted liver was examined grossly according to a local protocol, which included taking samples from each segment and from any nodular lesions differing from the surrounding cirrhotic nodules in terms of size, color, texture or bulging. The HCC patients included 12 males (mean age 61 years, range 58-65). In multifocal HCC (12/20 cases) only the largest cancer nodule was considered.

As detailed in Table I, 95 nodular lesions were investigated. Out of the 13 DN, seven were LG and six HG, all but two of them coexisting with HCC. Twenty cases of HCC (Grade 1: 6; Grade 2: 10 and Grade 3: 4) were included. Seven liver tissue samples obtained from seven normal organs rejected for transplantation (potential donors revealing incidental extra-intestinal malignancies) served as controls.

The tissue samples were fixed in formalin, embedded in paraffin and stained with hematoxylin and eosin, and van Gieson stain for fibrosis. Nodular lesions were classified according to internationally-accepted criteria (22-24).

Micronuclei and broken egg nuclei assessment. A representative section from each tissue sample was stained for DNA using the Feulgen method, without fast green contrast (21). The slides were analyzed by two of the authors (MF and LG) using the Cyres system (Zeiss, Jena, Germany), which consists of a conventional optical microscope connected to a monitor *via* a 3CCD color camera (JVC, Tokyo, Japan). A green interferential filter (549±10λ) (Schott, Mainz, Germany), set at the highest specific adsorption for the stain involved, was used to maximize counting accuracy. All the counting was conducted with a ×100 lens and the final images were analyzed at ×1,000 magnification.

The following accepted criteria (12, 21, 25) were applied for MNi assessment: round or oval shape and stain intensity equating to that of the main nucleus; diameter $\leq 1/16$th of that of the main nucleus in a mononucleated cell, or $\leq 1/3$rd in a binucleated hepatocyte; no contact with the main nucleus and the MNi membrane clearly separate from that of the main nucleus. Only MNi in phenotypically vital, not overlapping hepatocytes were considered and any refractive material was excluded. In all cases, at least 50 randomly-selected fields (corresponding to 2,000-2,500 hepatocytes) were analyzed. In accordance with the current literature, the results were expressed as micronucleated cells per 1,000 hepatocytes (MNi/1000Hep). Cells with two or more MNi were counted as a single micronucleated cell.

Table I. *Prevalence of micronucleated (MNi) hepatocytes and broken eggs (BE) in each histological category (the number in each category is also shown).*

Histology (number of samples)	MNi/1,000Hep[a] mean±SD (median)	BE/1,000Hep[b] mean±SD (median)
Normal liver (7)	4.50±0.76 (4.89)	0.61±0.36 (0.75)
Cirrhotic nodule (49)		
HCC–ve (26)	10.31±3.04 (9.72)	14.01±4.70 (14.38)
HCC+ve (23)	14.82±4.70 (14.38)	18.03±5.76 (18.99)
Large regenerative nodule (13)		
HCC–ve (6)	10.91±2.37 (11.94)	12.72±1.64 (12.26)
HCC+ve (7)	12.70±2.99 (12.46)	15.47±3.44 (15.80)
Dysplastic nodule (HCC+ve and HCC–ve) (13)	17.84±4.74 (18.64)	17.01±4.43 (16.39)
Hepatocarcinoma (20)	21.30±4.87 (22.97)	20.75±5.89 (21.74)

[a]Micronucleated hepatocytes in 1,000 observed hepatocytes; [b]broken egg nuclei in 1,000 observed hepatocytes; HCC–ve: lesion not coexisting with HCC; HCC+ve: lesion coexisting with HCC.

The frequency of broken egg (BE) nuclei was also considered in all the samples (21, 25). BE were considered as a fragmented (but attached) nucleus, the smallest of which corresponded to one third of the diameter of the larger nucleus. BE counts were expressed as BE per 1,000 hepatocytes (BE/1,000Hep).

Intra- and inter-observer (MF and LG) consistency in MNi and BE counts (calculated using the *k*-statistic) was tested in a subset of 10 randomly-selected cases (including normal liver - NL, CN, LRN, DN, and HCC). The *k*-values for intra- and inter-observer consistency were 0.87 and 0.85, respectively, for MNi and 0.88 and 0.84, respectively for BE.

Immunohistochemical study. The immunohistochemical analysis was conducted using a standardized avidin-biotin complex (ABC) method in an automatic system (Benchmark XT; Ventana, Tucson, AZ, USA). Hepatocyte proliferation was assessed by applying monoclonal Mib-1 antibody (Dako, Glostrup, Denmark; 1:50 dilution) and the results were expressed as the percentage of positive nuclei in 10 randomly-selected high-power fields (hpf). The p53 protein, expressed as the percentage of positive nuclei in 10 randomly-selected hpf, was assessed using pre-diluted monoclonal anti-p53 antibody (Immunotech, Marseilles, France). Immunohistochemical analysis was performed jointly by two of the authors (MG and MF), blinded to any clinical details and the results of MNi/BE assessment.

Statistical study. MNi/BE frequencies were compared between all the groups of nodular lesions. MNi/BE occurrence was correlated with patient age and gender, viral data (type of infection, HCV genotype), the histological parameters (number and size of HCC nodules, HCC grade) and the immunohistochemical parameters (Mib-1, p53).

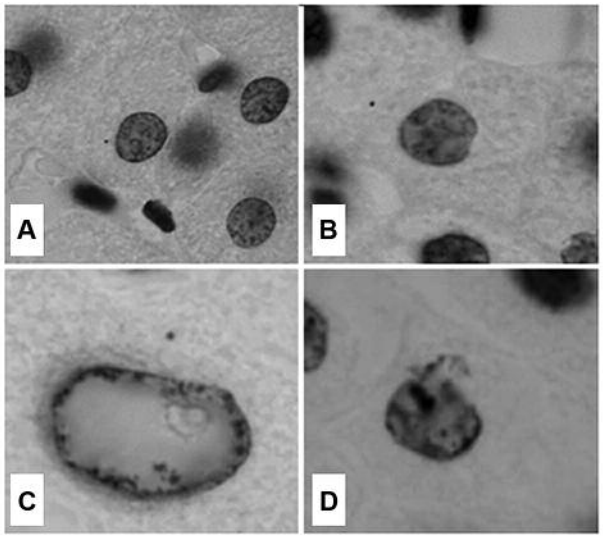

Figure 1. *Micronucleated hepatocytes in cirrhosis (A, B) and in a dysplastic nodule (C). A broken egg nucleus in cirrhosis (D) (Feulgen's stain; original magnification ×500).*

Table II. *Immunohistochemical expression of Mib1 and p53 in each histological category (the number in each histological category is also shown).*

Histology (number of cases)	Mib-1 mean score[a] ±SD (median)	p53[b]	p53 mean score[c] ±SD (median)
Normal liver (7)	0.50±0.46 (0.25)	2/7	0.43±0.79 (0)
Cirrhotic nodule (49)			
HCC–ve (26)	2.00±1.98 (1.13)	1/26	0.04±0.20 (0)
HCC+ve (23)	1.88±1.98 (1.25)	0/23	0.04±0.21 (0)
Large regenerative nodule (13)			
HCC–ve (6)	1.46±1.04 (1.38)	0/6	0
HCC+ve (7)	1.11±1.01 (0.75)	1/7	0.14±0.38 (0)
Dysplastic nodule (13)	2.18±2.39 (1.94)	0/13	0
Hepatocarcinoma (20)	33.68±40.29 (23.63)	6/20	8.50±18.99 (0)

[a]Expressed as the percentage of positive nuclei in 10 randomly-selected high-power fields (hpf); [b]p53 immunostaining positive cases; [c]Expressed as the percentage of positive nuclei in 10 randomly-selected hpf; HCC–ve: lesion not coexisting with HCC; HCC+ve: lesion coexisting with HCC.

The Mann-Whitney *U*-test, Spearman's rank, Kruskal-Wallis and Wilcoxon tests for paired data, and the modified Kruskal-Wallis non-parametric test for trend were used, as appropriate. Values of $p<0.05$ were considered significant. The *k* coefficient for pairs of observers was interpreted in accordance with the Landis and Koch benchmarks (26).

The diagnostic value of MNi was assessed by calculating the area under the receiver operating characteristic curve (AUC) and its corresponding 95% confidence interval (CI): AUC=0.5-0.7 no discrimination; 0.7≤AUC<0.8 acceptable discrimination; AUC≥0.8 excellent discrimination (27).

The statistical analysis was performed by one of the authors (LG) using STATA software (Stata Corporation, College Station, Texas, USA).

Results

Micronuclei and broken egg nuclei analysis. MNi and BE (Figure 1) were detected in all the analyzed samples in both the study and the control groups, and their frequencies were statistically associated with each of the different types of nodular lesion ($p<0.001$). MNi/1,000Hep and BE/1,000Hep were also significantly more frequent in all the nodular lesions considered than in the normal liver tissue (both $p<0.001$), with no overlap (Table I).

The number of MNi hepatocytes and BE nuclei significantly increased from CN to HCC (Kruskal-Wallis test for trend; $p<0.001$ and $p=0.002$, respectively).

The CN contained significantly fewer MNi/1,000Hep than the LG- or HG-DN ($p=0.011$), or the HCC ($p<0.001$). The prevalence of MNi was also significantly lower in the LRN than in the LG- or HG-DN ($p=0.020$), or the HCC ($p<0.001$). No difference emerged in MNi frequency between LG- and HG-DN (p=ns; probably due to the small number of cases involved), so the LG- and HG-DN cases were considered as a single group. Micronucleated hepatocytes were significantly more frequent in the CN and LRN coexisting with HCC than in the CN and LRN from HCC-free cases ($p=0.001$).

The CN and LRN contained significantly fewer BE/1,000Hep than the DN ($p=0.008$), or the HCC ($p=0.007$). BE nuclei were significantly more frequent in the CN and LRN coexisting with HCC than in the CN and LRN from HCC-free cirrhotic cases ($p=0.011$).

ROC analysis was performed to determine a cut-off for MNi and BE frequencies for discriminating between CN and LRN associated or unassociated with HCC. The MNi frequency was the best parameter for predicting CN/LRN associated with HCC. The AUC for MNi was 0.78 (CI 95%: 0.644-0.886). A cut-off of 12.34 MNi/1000Hep proved the best for an acceptable discrimination, revealing a 70% sensitivity and 72% specificity.

The prevalence of MNi and BE nuclei was significantly associated with size ($p<0.001$ and $p=0.001$, respectively) and number ($p<0.001$; $p=0.002$, respectively) of HCC nodules. In each histological category, the prevalence of MNi/BE revealed no significant relationship with etiology (HBV- or HCV-related infection, or HCV genotype).

p53 and Mib1 immunohistochemistry. Table II shows the Mib1 and p53 expression in all the lesions considered. Mib1 expression increased progressively from cirrhosis to cancer

($p<0.001$) and was significantly associated with the frequency of both MNi hepatocytes and BE nuclei ($p=0.008$, and $p=0.009$, respectively).

In HCC, p53 overexpression was detected in 6/20 cases (30%); in the cases showing p53 overexpression, the positive nuclei ranged from 10% to 80%. Both MNi and BE nuclei were significantly associated with p53 overexpression ($p=0.031$ and $p=0.043$, respectively). Only 1 out of 49 CN and 1 out of 13 LRN revealed any p53 immunoreactive nuclei (less than 5% of the hepatocytes in each case).

Discussion

Micronucleated hepatocytes were consistently found in significantly lower numbers in normal liver than in any of the types of non-neoplastic and neoplastic liver nodules; a frequency of ≤4.50±0.76 MNi/1,000 hepatocytes unequivocally identified normal liver tissue in this series.

A major finding of this study was that both MNi and BE (two phenotypic expressions of chromosome instability) significantly and progressively increased throughout multistep liver carcinogenesis, clearly demonstrating the importance of genotoxic damage in viral hepatitis.

In keeping with data from De Almeida *et al*. (21) and other studies (4), cirrhotic and large regenerative nodules revealed no significant differences in MNi prevalence, supporting the "non-neoplastic" biology of LRN. Both CN and LRN coexisting with HCC showed significantly more MNi and BE, however, than equivalent lesions occurring in HCC-free livers, possibly meaning that the MNi+ve/BE+ve phenotype identifies a subset of cirrhotic cases prone to, or already with concomitant cancer. A cut-off of 12.34 MNi cells achieved 72% specificity in identifying CN/LRN coexisting with HCC. The potential clinical impact of this finding would warrant prospective studies on the (differing) cancer risks associated with MNi in liver cirrhosis.

The highest frequency of MNi cells was observed in the DN and the HCC, but the difference between them was not significant. This is consistent with several studies indicating that DN are (neoplastic) precursors of HCC, with which they share a similar molecular (3, 4, 28) and histological (29-31) profile.

At no phenotypic levels did any differences in the number of micronucleated hepatocytes emerge in relation to HBV or HCV etiology, thus suggesting a similar genotoxic exposure (related to both necroinflammatory lesions and/or high mitotic activity) in virus-related liver oncogenesis. This virus-type-independent pathway, however, does not rule out the possibility of concurrent virus-related events, as demonstrated *in vitro* by MNin HepG2 cells occurring after HBV x protein (HBx) transfection (18, 32). Concerning HCV, *in vitro* experiments proved that viral infection may interfere directly with the mitotic apparatus, whose dysfunction is among the mechanisms leading to MNi/BE formation (33, 34).

TP53 deregulation was confirmed as being a late event in liver carcinogenesis, significantly associating the protein overexpression with the most advanced stages (*i.e*. DN) of neoplastic transformation (35-37). Inefficiency of the p53 check-point would allow a genetically unstable MNi+ve/BEs+ve cell population to survive and expand (20, 38).

In conclusion, both MNi and BE (both markers of chromosome instability) progressively increase over the course of human liver carcinogenesis. This study further supports the biological similarity of dysplastic nodules and full-blown liver cancer. Prospective studies should explore the predictive value of the MNi+ve/BE+ve phenotype in identifying cirrhosis already coexisting with, or at high risk of developing HCC.

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