Potential ultrastructure predicting factors for hepatocellular carcinoma in HCV infected patients


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Original Articles

Potential ultrastructure predicting factors for hepatocellular carcinoma in HCV infected patients


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ABSTRACT
Hepatitis C virus represents one of the rising causes of hepatocellular carcinoma (HCC). Although the early diagnosis of HCC is vital for successful curative treatment, the majority of lesions are diagnosed in an irredeemable phase. This work deals with a comparative ultrastructural study of experimentally gradually induced HCC, surgically resected HCC, and potential premalignant lesions from HCV-infected patients, with the prospect to detect cellular criteria denoting premalignant transformation. Among the main detected pathological changes which are postulated to precede frank HCC: failure of normal hepatocyte regeneration with star shape clonal fragmentation, frequent elucidation of hepatic progenitor cells and Hering canals, hepatocytes of different electron density loaded with small sized rounded monotonous mitochondria, increase junctional complexes bordering bile canaliculi and in between hepatocyte membranes, abundant cellular proteinaceous material with hypertrophied or vesiculated rough endoplasmic reticulum (RER), sequestrated nucleus with proteinaceous granular material or hypertrophied RER, formation of lipolysosomes, large autophagosomes, and micro-vesicular fat deposition. In conclusion, the present work has visualized new hepatocytic division or regenerative process that mimic splitting or clonal fragmentation that occurs in primitive creature. Also, new observations that may be of value or assist in predicting HCC and identifying the appropriate patient for surveillance have been reported. Moreover, it has pointed to the possible malignant potentiality of liver stem/progenitor cells. For reliability, the results can be subjected to cohort longitudinal study.

Introduction
Hepatocellular carcinoma is an aggressive disease with poor outcome [1,2]. It is the fifth most diagnosed cancer and the second cause of cancer death in men all over the world [1,3]. Chronic hepatitis C virus (HCV) infection is considered a leading risk factor for hepatocellular carcinoma worldwide [4–7]. It accounts for 20% of all HCC [8]. Despite the development of effective antiviral treatment, it is advised that treated patients with advanced fibrosis or cirrhosis undergo life-long screening for HCC [9–11]. Curative treatments are accessible on condition that HCC is perceived early and as long as the liver function is preserved [12,13]. Thus, population screening to identify patients with cirrhosis and subsequently thorough surveillance for HCC are mandatory to decrease related mortality [3,14,15]. Nowadays, screening of hepatic nodules by radiologic imaging techniques reveals great progress. Meanwhile, the lesions that do not exhibit classical imaging features still represent real challenge [16,17]. It was reported by Roncalli et al. [18] that imaging features of regenerative and dysplastic hepatocellular nodules between 0.5 and 1.5 cm are rarely diagnostic, and they are usually biopsied. Many studies tackled the histopathological differentiation of hepatic nodules comprising large regenerative nodule, low and high grade dysplastic

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lesions as well differentiated hepatocellular carcinoma [18–21]. Also, electron microscopic study of HCC was undertaken by many authors in experimentally induced HCC [22,23] or in human biopsies [24–27]. Meanwhile, no specific histopathological criteria were postulated by these studies as a predictor of malignancy.

The aim of this work is to compare ultrastructural pathological changes which occur in liver tissues throughout the development of experimentally gradually induced hepatocellular carcinoma in mice with the liver specimens of surgically resected human hepatocellular carcinoma over-existing HCV; the corresponding surgical safety free tumor resection margin; chronic hepatitis C with nodular cirrhosis; and regenerative nodules. The emphases are to identify at an ultrastructural level any morphological difference between the early stages of experimentally induced HCC and the pathological changes occurring in human HCC and the potential premalignant studied lesions. In the attempt to depict criteria denoting or predicting malignant transformation. Thus, we can identify the patients at risk who should have a closer follow-up and we can guaranty early diagnosis of malignancy with a more promising curative rate.

Materials and methods

The materials of this study consisted of 63 liver biopsies processed for electron microscopic examination. The source of these liver biopsies was as follow:

1. Twenty ultrasound guided liver core biopsies

They were collected from patients with chronic hepatitis positive for serum HCV RNA. They were of stage F4 compensating hepatic cirrhosis according to the METAVIR scoring system [28]. They were selected from the patients consecutively admitted to the hepat-gastroenterology department of Theodor Bilharz Research Institute hospital between the periods of December 2013 and January 2015. The exclusion criteria of the involved patients were the presence of any additional cause of chronic liver diseases; following any regimen for anti-HCV, antifibrotic or immunosuppressive therapy; the presence of any concomitant general diseases as rheumatoid arthritis, autoimmune disease or hepatocellular carcinoma. Liver cirrhosis was confirmed on the basis of clinical examination, computerized tomography (CT) scan and histopathological examination of the liver biopsies processed for light microscopic examination. All the cases revealed cirrhotic nodules except that five cases showed criteria of regenerative nodules. This differentiation was done according to the International Working Party (IWP) criteria [29].

2. Twenty-eight specimens from the surgically resected HCC and the corresponding tumor free surgical margin.

They were harvested from 14 patients having well circumscribed solitary tumor mass, undergoing partial surgical liver resection. The patients had pre-existing HCV infection and did not have any associating causes of chronic liver diseases, or general diseases. They were not exposed previously to antiviral, immunosuppressive, or any HCC treatment.

The liver specimens were harvested from the tumor mass and the corresponding free tumor resection margin. The preoperative diagnosis of HCC was based on triphasic multislice CT. While postoperative diagnosis of primary HCC and histopathological changes of free tumor resection margin depended on light microscopic examination of paraffin embedded resected liver specimens. The included samples for electron microscopic examination were nine early well differentiated HCC < 2 cm in maximum diameter and five progressed HCC. They have trabecular patterns with occasional pseudo acinar configuration. The specimens from the safety free tumor resection margin revealed criteria of liver cirrhosis with often high grade dysplastic nodular changes (HGDN). Histopathological diagnosis and classification of the tumor were performed according to the international consensus group for hepatocellular neoplasia [30] (Figure 1A).

3. Twelve liver samples collected from three groups of male albino mice Balb C injected with the hepatocarcinogenic reagent dimethylnitrosamine (DEN) [23,31]. Also, three liver samples from a normal control group of three mice were included in this study.
A new experimental mice model of hepatocarcinogenesis was accomplished by weekly intraperitoneal injection of dimethylnitrosamine (DEN) at a dose of 50 mg/kg for 16 weeks. A group of four mice each was sacrificed at the interval of 4, 8, and 16 weeks. Lesion progress up to the onset of HCC was inspected grossly and microscopically. The mice breeding and feeding were maintained under standard conditions at the Schistosome Biological Material Supply Centre (SBSC) of the Theodor Bilharz Research Institute (TBRI), Giza, Egypt. All manipulations performed with the animals were in the frame of the ethical guidelines for care of laboratory animals.

Grossly the mice liver revealed congestion and enlargement at the fourth week from the beginning of DEN injection. The mice liver showed diffuse nodular pattern at the 8th week of injection. The nodules were about 1mm in diameter. Protruded gross was seen at the 16th week. It was more than 4 mm in diameter (Figure 1B). Light microscopic examination of liver samples of 4 weeks exposed mice revealed cellular hyperplasia...
with low grade dysplastic cellular foci. The 8 weeks treated mice showed high grade dysplastic nodules. Sixteen weeks exposed mice to DEN revealed HCC nodules (Figure 1C).

Specimens for light microscopic examination were fixed in 10% buffered formalin and processed until embedded in the paraffin block. Four μm thick sections were stained with hematoxylin and eosin and Masson trichrome stain. Specimens for electron microscopic examination were divided into tiny pieces of 1 mm³ and fixed in 4% buffered glutaraldehyde in 0.2 M cacodylate then washed in an equal volume of 0.3 M cacodylate with sucrose 0.4 M. Samples were post fixed in 2% osmium tetroxide in distilled water, washed in water then dehydrated in ascending ethyl alcohol concentration, infiltrated, and embedded in Epoxy resin then polymerized at 60 °C. Semithin sections were prepared from the polymerized gelatin capsule using Ultracut R ultramicrotome (Leica, Vienna, Austria). The midzonal liver area was the site of choice for performing ultrathin sections. Semithin sections were stained with methylene blue and Azur II. Ultrathin sections were contrasted with uranyl acetate and lead citrate. The sections were examined using Philips EM 208S at an accelerating voltage of 80 kW.

The experimental work and human samples collection were conducted after approval of Theodor Bilharz Research Institute Review Board for ethics (TBRI RB). Enrolled patients in this study have been asked to write an informed consent before being subjected to any medical intervention. For animal manipulations, TBRI RB follows the ethics approved by the Ethical Committee of the Federal Legislation and National Institutes of Health Guidelines in the USA.

Results

The main histopathological characteristic of the different studied experimental and human lesions at the level of light microscope was summarized in the form of the figure legend.

Ultrastructural morphological characteristics of experimentally induced HCC

The ultrathin liver sections prepared from mice exposed to DEN for 4 weeks revealed hepatocytes with different electron density; hepatic stellate cells loaded with fat droplets located intercellularly and in the sinusoidal area; hypertrophied rough endoplasmic reticulum (RER) sequestrating mitochondria with fragmented and disoriented cristae. Some of these mitochondria underwent degeneration and liberated their cristae in the cytoplasm in the form of small vesicles (Figure 2). The electron dense hepatocytes showed an increase in uniform small sized round mitochondria, enrichment in RER arranged in parallel arrays, an increase in cytoplasmic proteinaceous material and free ribosomes. The cells disclosing a decrease in electron density revealed excess fat deposition and few cellular organelles or signs of degenerative changes. Also, it was depicted in the examined sections hepatocytic regenerative changes with the formation of a cellular rosette, loss of hepatocytes polygonal configuration, nuclei with irregularly indented membrane and prominent nucleoli.
The liver sections of mice exposed 8 weeks to DEN were characterized by frequent detection of Hering canals and inter hepatocytic bile ductules with undeveloped luminal microvilli. The bile duct-like cells forming the bile ductules or sharing in the formation of Hering canals with hepatocytes were rich in cytoplasmic organelles. They had elongated oval nuclei with irregular nuclear membrane and dispersed chromatin condensation in comparison with typical bile duct cells or cholangiocytes. Oval cells were seen in close vicinity to bile ductules and Hering canals. They appeared small oval cells of about 5 microns with poorly developed cytoplasmic organelles. Their large oval nuclei showed clumped heterochromatin. Bile canaliculi, not occasionally as normal, were bound with more than three or more hepatocytes. Some revealed amorphous inclusion or cellular organelles in their lumens. Desmosomes bounding the bile canaliculi were increased. Intermediate hepatic cells were often seen within hepatocytes cords or clusters. They appeared larger than the oval cell but smaller than the mature hepatocytes. Their nuclei were rich in euchromatin and their

Figure 1C. a and b: Liver sections of 4 weeks exposed mice to DEN injection show different degree of cellular hyperplasia. Note in figure b the irregularity in cellular cytoplasmic density and microacinar formation (arrow) (Haematoxylin and eosin 20×). –c and d: Liver sections of 8 weeks exposed mice to DEN injection reveal high grade dysplastic changes. Note the irregular hyperchromatic nuclei in figure c, and the clear nuclei with prominent nucleoli as well as intraparenchymal bile ductules (arrow) in figure d (Haematoxylin and eosin 20×). Both sections show crowded hypercellularity of irregular size small cell changes, with alternating different cellular density. –e and f: Liver sections of 16 weeks exposed mice to DEN injection reveal HCC of thick trabecular cell plates with hyperchromatic irregular sized nuclei and increased vascularity (Haematoxylin and eosin 20×). With the progress of the lesion, note the turnover of haematoxylin and eosin stained hepatocytes from acidophilic hepatocytic cytoplasm seen in figure (a) to basophilic cytoplasm which reaches its maximum density in figure (f).
cytoplasm disclosed more developed cell organelles. They appeared less electron dense in comparison with mature hepatocytes. They may constitute one or more cells in bile canaliculi boundary. Lipolysosomes or liposomes were often seen in hepatocytes and occasional extravasated RBCs were detected in the examined fields (Figure 3). Beside these previously mentioned hepatocytic morphological changes which characterized this stage of DEN exposure, it was depicted foci of small sized hepatocytes or trabeculae of more than two cells thick. These cells disclosed an increase in nearly uniform sized mitochondria, intracytoplasmic proteinaceous material, and large bizarre shape nuclei with prominent nuclear pores. The nuclei had an irregular nuclear membrane with prominent margined nucleoli or pseudonuclear inclusions.

The liver sections of mice sacrificed after being injected intraperitoneally weekly by 50 mg/kg of DEN for 16 weeks were characterized by the detection of newly formed unpaired arteries with their peculiar protruding endothelial lining, abortive elastic lamina and smooth muscle layer; absence of Hering canals; occasional disclosure of bile ductules in between hepatocyte clusters or hepatic trabeculae; active division of intermediate hepatocytes and the visualization of star shape enucleated cytoplasmic cellular masses having dendritic projections. Moreover, nuclear changes were prominent with an increase in the nuclear-cytoplasmic ratio. The nuclei showed indentation, occasional pseudo-cytoplasmic inclusion, multiple margined nucleoli, and prominent nuclear pores. Some pores had an unclear diaphragm. The nuclear chromatin was formed mainly of euchromatin with condensation of heterochromatin in the form of clumps at a regular interval along the inner side of the nuclear membrane. The circumference of many nuclei was encircled by condensed fine granular proteinaceous material, free ribosomes, microtubules, and microfilaments. The hepatocytes showed hyperplastic hypertrophied rough endoplasmic reticulum arranged parallel in close contact with the increased uniform, nearly rounded of equal size mitochondria. Sequestrated

Figure 2. Liver sections of four weeks exposed mouse to DEN. -A: The liver section shows Intercellular hepatic stellate cells (S) loaded with fat droplets and hepatocytes (H) with different electron density. -B and C: Electron micrographs reveal hepatocytes with increase electron density more than twice that in the vicinity. The electron dense cells (asterisks) illustrate an increase in free ribosomes, proteinaceous material, small size mitochondria and rough endoplasmic reticulum. The electron lucent cells (H) of micrograph B shows degenerative changes While in C reveals fluffy diffuse fat deposition and few cell organelles. -D: Sequestrated mitochondria with hypertrophied rough endoplasmic reticulum (double arrow). Note the degenerated mitochondrion with the liberation of its content in the form of microvesicles (arrow).
mitochondria with RER were a common finding. Also, distended RER cisternae loaded with a proteinaceous material were seen liberated from degenerated cells. The hepatocytes did not maintain their polygonal configuration and they revealed an increase in electron density. They showed well-developed Golgi apparatus, an increase in cytoplasmic proteinaceous material, autophagosomes, lipolysosomes, and fat deposition mainly in the form of microvesicular droplets. Moreover, fat droplets infiltrated with microfilaments or dissected with electron dense condensed cytoplasmic microfilaments were perceived in the examined sections. The intercellular spaces between tumor cells were often filled with a fibrin-like material. The detected sinusoidal spaces were capillarized and showed hypertrophied Kupffer cells loaded with residual lysosomes (Figures 4 and 5).

**Characteristic of surgically resected HCC**

The examined ultrathin sections of surgically resected HCC revealed comparable ultrastructural cellular criteria to what seen in the 16 weeks DEN-exposed mice. Small irregular sized hepatocytes were arranged in trabeculae of more than two cells thick or forming pseudo acini. The hepatocytes appeared either as clear electron lucent cells displaying minimal cellular organelles and diffuse fat deposition; or electron dense cells showing proliferated rounded small size mitochondria with electron dense matrix, deposition of microvesicular fat droplets, increase cytoplasmic proteinaceous material and hypertrophied RER. Also, hepatocytes were configured as bizarrely shaped cells revealing few cytoplasmic cellular organelles and fat droplets traversed by a fibrillar material. This later tumor cell configuration was seen in progressive HCC. The tumor

**Figure 3.** Liver sections of eight weeks exposed mouse to DEN. – A: Bile ductule (B) seen in between hepatocytes (H), undergoing formation by the assembly of newly formed bile duct like cells rich in cytoplasmic organelles. Oval cell (O) is seen in the vicinity of its basement membrane. B: Canalicular ductular junction or Hering canal formed by the junction of hepatocytes (H) with the biliary epithelial cell (B). The formed lumen (L) shows rudimentary microvilli. Note the oval cell (O) in close contact with the canal. – C: Bile canaliculi bordered by hepatocytes (H) and intermediate hepatocyte of progenitor cell origin (IHP) showing oval nucleus perpendicularly oriented to the lumen (L) with increase in cell junctions at the margin of the lumen. – D: Hepatocyte (H) shows small size mitochondria with sparse cristae, Hyperplastic disrupted rough endoplasmic reticulum studded with ribosomes, increase cytoplasmic proteinaceous material, liposomes or lipolysosomes (arrow), free ribosomes, nucleus (N) with prominent pores (curved arrow) in between there are heterochromatin clumps.
Figure 4. Liver sections of 16 weeks DEN exposed mice. –A: Active intermediate hepatocyte of progenitor cell origin (IHP) in between hepatocytes showing replicated centrosomes (arrow) for the beginning of mitotic cell cycle. –B: Intermediate hepatic progenitor cells (IHP) with increase nuclear cytoplasmic ratio forming trabeculae of more than two cell thick. The nuclear chromatin is formed mainly of euchromatin. –C: The circumference of the nucleus is sequestered with an area of fine granular proteinaceous material, free ribosomes and microfilaments. The nuclear chromatin is formed mainly of euchromatin with regular heterochromatin condensation along the nuclear membrane demarcating nuclear pore. –D: Star shape cytoplasmic masses (M) with excess stunted processes.

Figure 5. Electron micrograph of Liver sections of 16 weeks DEN exposed mice. –A: Newly formed small vessels (arrow). The endothelial lining (E) protruding in the lumen mimic that of small arterioles. Meanwhile, the smooth muscle and elastic tissue are poorly developed. –B: Interhepatocytic small arteriole in a formation phase (arrow). Note the oval progenitor cell (O) constituting the endothelial lining which protrudes intraluminal; the elastic tissue and smooth muscle cell are not clearly determined. –C: Fat droplets (L) infiltrated with microfilaments and dissected with electron dense condensed cytoplasmic microfilaments. –D: Distended vesiculated rough endoplasmic reticulum loaded with proteinaceous material and microtubules (arrow).
cells revealed either monotonous, regular rounded nuclei formed of euchromatin with or without small size nucleoli or bizarre shape large irregularly indented nuclei showing dispersed clumps of heterochromatin with segregated and marginated nucleoli. The nuclei of binucleated cells lacked similarity. They were of different size and shape. Hepatocyte progenitor cells were often seen in between hepatocytes clusters or trabeculae. As well as constituent of the newly produced bile ductules in the form of cholangiocyte like cells. Sinusoidal like space, extravasated RBCs, Unpaired arteries with abortive elastic interna and smooth muscle, cannibalistic cells were detected in the examined sections (Figure 6). Apart from the previously mentioned morphological changes observed in HCC sections, which were as well distinguished at the level of light microscopy, it was depicted in the examined sections hepatocytes showing mouse mammary tumor virus-like in their cytoplasm. The later appeared as sequestrated mitochondria by the cisternae of RER. The mitochondria showed nearly homogenous electron dense matrix without cristae or revealed very few undeveloped cristae. The cisternae of RER showed deposited proteinaceous material at regular intervals along the outer membrane of the cisternae. Also, it was disclosed increase in junction complex adjoining cell membrane along the canalicular and lateral surfaces of adjacent hepatocytes and even between cellular microvilli, pseudo nuclear inclusions, autophagosomes showing RER or mitochondria, ribosomes studded vesicles, vesicles sequestering polyribosomes, degranulated RER vesicles, membrane-bound lipidic bodies or liposomes, detached cellular parts bounded by intact membrane, star shape cytoplasmic masses with extended processes seen detached from hepatic progenitor cell or malignant cells (Figure 7).

**Characteristic of surgical safety free tumor resection margin**

Prepared ultrathin sections from free tumor resection margin revealed foci of cellular changes comparable

![Figure 6](image_url)

Figure 6. Electron micrographs of liver sections demonstrating different morphological configuration of HCC. –A: group of small size hepatocytes of early well differentiated HCC showing increase nuclear cytoplasmic ratio arranged around vascular space (arrow) giving the picture of pseudo acinar pattern. –B: Malignant cells of early well differentiated HCC showing increased intercellular junction (arrow), nuclear chromatin consisted of euchromatin. –C: Progressive HCC showing enucleated cytoplasmic masses (M) with extended microvilli, mitochondria with electron dense matrix, many liposomes (arrow). –D: Group of small sized cells of progressive HCC showing bizarre shape nuclei with condensed heterochromatin along nuclear membrane seems to be the result of an uncontrollable division of intermediate hepatocytes of progenitor cell origin.
to the cells of HCC sections but without the revelation of small bizarre shaped cells. They displayed abnormal architectural configuration with pseudoacinar formation, thick liver cell plate more than three cells wide, and increase in the nuclear-cytoplasmic ratio. Hering canal, bile ductules, and bile canaliculi were often detected in this category of studied liver samples. This is comparable to the findings of eight weeks DEN-exposed mice. Hepatic progenitor cells were often depicted between hepatocytes, or as a constituent of bile canaliculi in the form of intermediate hepatocytes. Cholangiocyte-like cells formed intercellular bile ductules. Moreover, the examined sections revealed an increase in desmosomes like structure along the biliary pole of hepatocytes, frequent detection of kupffer cells and cellular debris in capillarized blood sinusoids, intracytoplasmic lipofuscin granules, residual lysosomes, and small RER vesicles not studded with ribosomes, and neovascularization seen in between the hepatocytic cords. Star shape cytoplasmic masses similar to that distinguished in the ultrathin sections of surgically resected HCC and experimentally induced HCC were distinguished in the examined sections (Figure 8).

**Electron microscopic examination of cirrhotic and regenerative nodules**

Cirrhotic nodules revealed abnormal architectural configuration. Hepatocytes were dissected by fibrous tissue or forming a rosette around vascular channel or bile canaliculus. Cellular regeneration showed autonomous cell proliferation. Binucleated cell disclosed nearly similar nuclei in shape and size. Nuclei with prominent nucleoli were depicted in most the examined hepatocytes. The mitochondria in cirrhotic nodules were of irregular size and shape with electron dense matrix. The RER showed hypertrophy with increased proteinaceous material in its cisternae. It showed occasional condensation around the nuclei or in close relation to mitochondria. The cytoplasm was occupied with diffuse fat deposition or large fat droplets. Tight junction is preserved

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**Figure 7.** Electron micrographs of liver sections demonstrating some changes associating HCC. –A: Detached star shape cytoplasmic mass (M) with protruding microvilli from intermediate hepatocyte of progenitor cell (IHP) and Hepatic progenitor cell (HP). –B: Sequestrated mitochondria by rough endoplasmic reticulum. The mitochondria show very sparse cristae or devoid of cristae and the outer surface of RER reveals regular electron dense deposits (arrow) different from ribosomes, mimic the core and capsid of mouse mammary tumor virus. –C: Well demarcated Cytoplasmic cellular mass (arrow) shows multiple autophagic vacuoles containing electron dense filamentous and proteinaceous material. –D: Well-formed unpaired small artery showing peculiarly shaped endothelial cells.
between adjacent hepatocytes at the edge of bile canaliculi. No Hering canal or intercellular bile ductules were disclosed in the examined sections. (Figure 9).

The five cases diagnosed at the level of light microscopy as regenerative nodules revealed apart from the previously reported changes seen in cirrhotic nodules, they displayed star shape enucleated cytoplasmic masses with processes seen mainly along the hepatocytes sinusoidal pole. They appeared detached from viable hepatocytes with intact active nuclei rich in euchromatin. These cells seem to undergo specific kind of primitive cell division or splitting. These hepatocytes did not disclose any criteria of apoptotic changes or necrosis. Small sized regenerating cells arranged concentrically and look like cholangiocytes were disclosed in between mature hepatocytes. Some of these cells were devoid of a nucleus. Also, hepatic stellate cells were frequently detected intercellularly or along blood sinusoids meanwhile hepatic progenitor cells were occasionally disclosed (Figure 10).
Figure 9. A and B: Electron micrographs illustrating ultrastructural morphological changes in the cirrhotic liver. –A: Hepatocyte shows irregular sized mitochondria with electron dense matrix. The chromatin of the nucleus is formed of euchromatin. –B: Group of hepatocytes, separated by fibrillary and proteinaceous deposit, show fat droplet deposition, the characteristic pyknotic mitochondria of hepatitis. –C and D: Electron micrographs illustrating ultrastructural morphological changes of the regenerative nodule in cirrhotic liver. C: Hepatic stellate cell loaded with fat droplets. –D: Hepatocyte shows sequestrated nucleus with rough endoplasmic reticulum, small rounded mitochondria and hypertrophied rough endoplasmic reticulum studded with ribosomes.

Figure 10. Electron micrographs of liver sections of the regenerative nodule demonstrating the star shape cytoplasmic masses with extending processes. –A: Cytoplasmic mass nearly taking star configuration and many are seen in between hepatocytes. –B: Detached part (M) from a hepatocyte (H) forming bile canaliculus with the mother cell (curved arrow). Note the site of cleavage (arrow) (TEM: x5600). –C: Liberated cytoplasmic star shape masses (M) with extended processes showing electron dense small round and rod shape inclusion mimic mitochondria (TEM: x5600). –D: The hepatocyte split up into small pieces (arrow) showing electron dense mitochondria and have the ability to produce small processes for mobility. Note the nucleus (double arrows) rich in euchromatin with intact nuclear membrane and prominent nucleolus; the mitochondria appear small in size and electron dense (TEM: x4400).
The main histomorphological differentiating features of the studied lesions at the level of light microscope and electron microscopy was summarized in Tables 1 and 2. Also, the main proposed criteria for predicting malignant transformation or assisting in the diagnosis of inconclusive liver biopsy examined at the level of the light microscope by the use of electron microscopy was illustrated in Tables 2 and 3. The absent and the present morphological feature symbolized by − and + reported in the tables assist in the diagnosis of the corresponding lesion and its differentiation from the other studied lesions. The uncommon morphologic feature symbolized by ± in the table points to the criteria when present predicts malignant transformation, if it is equally mentioned as criteria of malignant lesion.

**Discussion**

To our knowledge, this is the first ultrastructural work which tackles a comparative study between gradually induced HCC in experimental mice model versus human surgically resected HCC and potential premalignant nodular liver with the coexistence of HCV infection. This was done with the prospect, to deduce the changes that precede the development of frank HCC and which can assist in its prediction. This in turn, can be of

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**Table 1. Main histomorphological differentiating features of the studied lesions at the level of light microscope.**

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<th>RN</th>
<th>HGDN</th>
<th>HCC</th>
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<td>Trabecular cell plates</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>more than 3 cells thick</td>
<td>−</td>
<td>−</td>
<td>±</td>
<td>+</td>
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<tr>
<td>Increase nuclear cytoplasmic ratio</td>
<td>−</td>
<td>−</td>
<td>±</td>
<td>+</td>
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<tr>
<td>Increase cell density</td>
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<td>more than twice the normal surrounding</td>
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<td>±</td>
<td>+</td>
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<tr>
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<td>Unpaired arteries</td>
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<td>−</td>
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<td>+</td>
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<tr>
<td>Ductal reaction</td>
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<td>+</td>
<td>±</td>
<td>+</td>
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<tr>
<td>Absence of portal tract</td>
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<td>−</td>
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<tr>
<td>Stromal invasion</td>
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<td>−</td>
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<td>Peripheral compressed hepatocytes</td>
<td>−</td>
<td>+</td>
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**Table 2. Main histomorphological differentiating features of the studied lesions at the level of Electron microscopy.**

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<td>Cellular atypia</td>
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<td>Hepatocytes show mitochondria of different size and shape</td>
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<td>±</td>
<td>−</td>
</tr>
<tr>
<td>Microvesicular fat droplets infiltrated by microfilaments</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Lipidosomes</td>
<td>−</td>
<td>−</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>(membrane bound lipidic material with centrally located electron dense deposit)</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hering canals formed with cholangiocyte like cells</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Kupffer cells</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Hepatic stellate cells</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Star shape enucleated cytoplasmic masses</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Frequent autophagosomes</td>
<td>−</td>
<td>−</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>Unpaired arteries</td>
<td>−</td>
<td>−</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>Mouse mammary tumor virus-like</td>
<td>−</td>
<td>−</td>
<td>±</td>
<td>+</td>
</tr>
</tbody>
</table>

**CN** = cirrhotic nodule; **RN** = regenerative nodule; **HGDN** = high grade dysplastic nodule; **HCC** = Hepatocellular carcinoma.

±: may be present; +: commonly present; −: absent
value in the distinction between early HCC and HGDN which still represents an important histopathological challenge especially in liver biopsy rather than resected tissue [18].

In the present work, the reported main ultrastructural pathological changes characterizing HCC (Figures 6A, B, D, and 7D) and cirrhotic liver on top of HCV (Figures 9A and B) were in agreement with IWP guidelines [29] and the data reported by many other authors [18,19,22,24,25,32–39]. Also, the ultrastructure findings of this study have substantiated that the progress of liver toxicity induced by Dimethylnitrosamine (DEN) to the development of HCC [31,40] elicits pathological changes comparable to changes seen in human hepatocellular carcinoma (Figures 4B and D; 5A, B, and D with 6C and D; 7A, C and D) and liver tissue taken from the safety free tumor margin after resection (Figures 3A–D; 4D; 5A and B with 8A–C, E and F). In addition, the present article highlighted some new ultrastructural observations which may represent pathological potential of HCC development.

The first remarked change in our experimental model of mice exposed for 4 weeks to DEN was the alternating electron dense and electron lucent area of liver parenchyma (Figures 2A–C) with the frequent detection of hepatic stellate cells intercellularly (Figure 2A). This different electron density of hepatocytes was reported as the morphological malignant sign of liver nodules denoted equally at the level of light microscope [18,19,20,25,40]. In the present work, the frequent detection of hepatic stellate cells in this early stage of experimental HCC induction and in the examined regenerative nodules of HCV (Figure 9C) infected patients supports the previously reported postulation of Yin et al. [41] that hepatic stellate cells have a profound impact in liver regeneration and cancer. Also, it was reported that mature hepatic stellate cells result in differentiation of liver stem/progenitor cells or oval cells into cholangiocytes [33,42,43]. Through either paracrine signaling pathways or cell–cell interaction [42–44]. This can justify the frequent detection of newly formed Hering canals and bile ductules in which cholangiocytes like cells formed part of their constituents in 8 weeks DEN-treated mice (Figures 3A and B) and the specimens of the free tumor surgical margin (Figure 8B). Conversely, Human HCC and 16 weeks DEN-exposed mice revealed occasional intercellular bile ductules but did not show Hering canal in the examined sections. Thus, this pathological transition may represent a pathological potential for HCC development.

It is worth noting, that our results regarding the location of oval cells or hepatic progenitor cells in the vicinity of Hering canals and bile ductules are consistent with the observations made by [45–47] and support the statement that oval cells or hepatic progenitor cells are the progeny of periductular stem cells [1,31,47,48].

By the 16th week of DEN exposure neovascularization and formation of unpaired or isolated arteries were disclosed (Figures 5A and B). This is consistent with the fact that malignant lesions upon growth become hypervascular and unpaired arteries within liver nodule are a specific feature of

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**Table 3.** Histopathological features which are considered by this study transitional stage or predictor for malignant transformation and recommend close patient follow up in inconclusive diagnostic biopsy at light microscopic examination.

<table>
<thead>
<tr>
<th>Relevant morphological criteria with high diagnostic value</th>
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<tbody>
<tr>
<td>1 – Clonal fragmentation of hepatocytes or star shape enucleated cytoplasmic mass</td>
</tr>
<tr>
<td>2 – Frequent detection of hepatic progenitor cell or dividing intermediate hepatocyte.</td>
</tr>
<tr>
<td>3 – Biliary tree changes: Hering canal showing cholangiocyte like cells. Bile ductule in between hepatocyte trabeculae. Bile canaliculi bordered with more than three hepatocytes or intermediate hepatocytes. Increase short intercellular not well formed junctional complex.</td>
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<tr>
<td>4 – Alternating electron light and electron dense hepatocytes showing abundant proteinaceous material.</td>
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<tr>
<th>Subsidiary morphological criteria with moderate diagnostic value</th>
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<tbody>
<tr>
<td>1 – Packed hepatocytes with uniform monotonous round small size mitochondria.</td>
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<tr>
<td>2 – Small round vesiculated degranulated RER filling the cell cytoplasm.</td>
</tr>
<tr>
<td>3 – Presence of cytoplasmic lipolysosomes, large autophagosomes and liberated large vesicles of RER distended with proteinaceous material.</td>
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<tr>
<td>4 – Loss of the diffuse appearance of fat deposition or demarcated microvesicular fat deposit.</td>
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<tr>
<td>5 – Lack resemblance of nuclei by RER or proteinaceous materials.</td>
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<tr>
<td>6 – Lack resemblance of nuclei of binucleated cells.</td>
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<tr>
<td>7 – Increase tight junction between hepatocytic membranes and cellular microvilli.</td>
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<tr>
<td>8 – Presence of cytoplasmic structure that mimics mouse mammary tumor virus.</td>
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</table>

**Neovascularization**

Undeveloped parenchymal vessels.

Unpaired arteries.
HCC [17,19]. Even, in the present work, the unpaired artery was displayed in three liver samples of surgical free tumor margin of progressive HCC (Figure 8C). Also, all liver specimen of free tumor surgical margin displayed foci showing cellular changes (Figure 8E) as in HCC specimens (Figures 6C and 7A) and the 16 weeks exposed mice to DEN (Figure 4D). This is in agreement with Okusaka et al. [49], Sasaki et al. [50] and kudo [40] who reported the presence of microscopic foci of malignant cells at the edge of hepatic tumor resection. Also, these findings reinforce the reported high recurrence rate of HCC which reach 40–75% with 5-year survival rate of less than 10% [51,52]. Thus, patients with resected tumor must pursue restricted follow-up regimen.

Oval cells or Hepatic progenitor cells were depicted in the groups of mice treated with DEN for the induction of HCC, in liver specimens of human HCC, surgically free tumor margin, and liver regenerative nodule. Moreover, actively dividing intermediate hepatic cells were depicted in 16 weeks experimentally exposed mice to DEN (Figure 4A). In the present work, hepatic progenitor cells or intermediate hepatocytes were not depicted in the cirrhotic nodule of HCV infected liver at the level of electron microscopy. Meanwhile, malignant intermediate hepatocyte like cells was seen in progressive human HCC specimens (Figure 6D). Based on this, we can assume that the visualization of hepatic progenitor cells or the actively dividing intermediate hepatic cells into liver sections at the level of electron microscopy is considered predicting factors for the progression to malignant transformation. It was reported that small cells in dysplastic foci which constitute the earliest premalignant lesions in hepatocellular carcinoma are formed mainly of progenitor cells and intermediate hepatocytes [53]. Moreover, many studies have drawn attention to the possible involvement of Hepatic progenitor cells in the process of hepatic tumorigenesis. [42,51,53,54]. It is generally accepted that cancer arises from the malignant transformation of stem cells [55], and, tumors revealing hepatic progenitor cell structures have a poorer prognosis and a higher recurrence rate versus tumors lacking these features [56,57]. In consequence, the frequent detection of hepatic progenitor cells in liver sections at the level of electron microscopy is considered by this study a transitional stage between benign and malignant microenvironment initiation predicting the swing to malignant transformation. Thus, Attention should be paid to the tumorigenic ability of hepatic stem progenitor cells in the liver therapeutic field.

In the present work, the studied specimens of regenerative nodules elucidated splitting or fragmentation of some hepatocytes into cytoplasmic masses (Figure 10D) taking nearly star shape appearance with extended processes. These hepatocytes did not show signs of apoptotic changes or necrosis. This may denote failure of hepatocyte regeneration as it is associated with the occasional detection of hepatic progenitor cells. It is reported that impairment of hepatocytes regeneration may trigger the expansion of stem/progenitor cells counterbalancing the inhibited regenerative ability of mature hepatocytes [58,59]. This hepatocyte fragmentation or splitting can be considered clonal fragmentation or kind of regeneration. This kind of primitive division or fragmentation looks like the division which occur in starfish. In another word, each fragment can develop to produce a new cellular structure or live as in autotomy, live independently. Especially, the separated part has the ability to move freely and seems contain electron dense mitochondria with replicated DNA.

Hence, based on the previously mentioned discussion and reported cellular morphological changes in this work there is increasing evidence that some electron microscopic hepatocytic criteria can be postulated by this study as predictor factors for malignant transformation in HCV-infected patients with cirrhosis. They can be summarized into four relevant and ten auxiliary or subsidiary criteria that can be considered as factors which may postulate carcinogenic potential. The four more relevant criteria proposed by this study are the detection of star shape cytoplasmic masses detached from progenitor cells or hepatocytes which may designate the impairment of normal cell regeneration or division and the resort to alternate cell replication (Figures 10D and 8E); the frequent elucidation of hepatic progenitor cells and dividing intermediate hepatocytes like cell in the examined sections (Figure 8D); biliary tree changes in the form of the frequent detection
of Hering canal (Figure 8B), bile ductules in between hepatocyte clusters or trabeculae, and bordered bile canaliculi with more than three hepatocytes or intermediate hepatocyte-like cells, with increase intercellular junctions (Figure 8A); and different electron density of hepatocytes. The ten subsidiary morphological criteria were equally observed in the early stages of experimentally induced HCC, in human HCC and specimens of free tumor surgical margin. Meanwhile, they were absent in the specimens of cirrhotic nodules. They comprise packed hepatocytes with uniform monotonous round small size mitochondria with medium or deep electron dense matrix (Figures 2C and 8A); small round vesiculated degranulated RER filling the cell cytoplasm; formation of lipolysosomes (Figures 3D and 8E); liberated cellular RER vesicles distended with proteinaceous material (Figure 5D); increase in autophagosomes (Figure 7C); dissected microvesicular demarcated fat droplets showing or not microfilament (Figure 5c); sequestration of the nuclei by RER or proteinaceous material (Figure 4C and 9D); lack similarity between newly formed nuclei; increase intercellular junctions between hepatocytic membranes or cellular projections (Figure 3C and 8A). It is worth noting, that these previously mentioned changes were reported by Ghadially [60] as criteria seen in tumor cells. The tenth postulated subsidiary criterion is the sequestration of mitochondria with RER cisternae (Figure 7B). The mitochondria showed electron dense homogenous matrix with absent or abortive cristae. The RER revealed regular proteinaceous deposit along the outer cisternal membrane, mimic mouse mammary tumor virus-like. This pathological figure was previously reported by Mansy et al. [61] in cases of HCV. They postulated its possible implication in the process of cellular transition to malignancy.

We believe that these assumed premalignant morphological criteria should not be considered apart from each other and be better evaluated using an initiated scoring system. This score includes two groups: the four relevant and the ten subsidiary observations reported in this study. Each criterion in the first group is presented by the figures 0 absent or 1 if present and each item of the second group is presented by the figures 0 absent or 1 if present. The evaluation of these criteria grades 0–22.

In conclusion, the present study has shed light on peculiar morphological hepatocytic alterations as a potential predictor of HCC. These morphological changes would help understand mechanistically what is happening in the process of hepatocarcinogenesis, which seems for reliability be subjected to cohort longitudinal studies. Also, the study has supported the previously reported speculation of the malignant potentiality of liver stem/progenitor cell and the impact of HSC on this process. Additionally, this article has drawn attention to a new hepatocyte fate or division, the hepatocytes fragmentation which needs further detailed study.

It is recommended that patients underwent surgical resected HCC or having a cirrhotic liver with regenerative nodules must be subjected to an appropriate surveillance. Importantly, if a biopsy is required, it must be subjected to light and adjunct electron microscopic examination with the application of molecular and immunohistochemical tumor markers analysis. These collaborative methods may offer an important contribution to predicting malignant transformation and realize accurate diagnosis.

**Author’s contribution**

Soheir Mansy obtained funding of the project 8k, put the idea of the article and the design of the human part of the work, performed microscopic examination and interpretation of the human and experimental enrolled samples, analyzed, and discussed the obtained results, wrote the manuscript. Eman El-Ahwany obtained funding of the project 111T, put the idea and the design for the experimental animal part of the work. Sarah Hassan harvested and screened the specimens of human HCC. Soheir Mahmoud injected and supervised mice breeding. Mona Zoheiry contributed to the supervision of the mice injection and breeding. Mohammed I Seleem, Amr Abdelaa, and Ahmed H Helmy provided the work with resected HCC and patient’s clinical data. Ahmed S. AbdelFattah and Moataz H Hassanein provided the work with...
the core liver biopsies and patient’s clinical data. All authors approved the manuscript.

Declaration of interest
The authors who have taken part in this work declared that they do not have any conflict of interest in regarding this manuscript.

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