The Role of HSP70, CD34 and Ki 67 Expression in Liver Cirrhosis and Hepatocellular Carcinoma, Immunohistochemical Study

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INTRODUCTION
In Egypt, hepatocellular carcinoma (HCC) is the second most common malignancy in males and the fifth in females. There was almost a twofold increase of HCC among chronic liver disease patients in Egypt in the past ten years with a significant decline of HBV and a slight increase of HCV as risk factors (El-Zayadi et al., 2005). Liver cirrhosis is an important risk factor of (HCC) with more than 80% of cases arising in the background of cirrhosis (Amarapukar et al., 2008). Distinguishing small HCC from other types of small focal lesions that occur in a cirrhotic liver can be difficult on the basis of morphologic features alone (Libbrecht et al., 2006). Hepatocyte proliferation in the cirrhotic liver promotes the development of HCC.
A few studies have explored the possibility of the use of proliferation markers as predictors of the development of HCC in the cirrhotic liver (Mary et al., 2006). Hepatocellular carcinoma (HCC) is the 6th most common cancer worldwide and the 3rd most frequent cause of death of cancer (Aileen, 2011). Hepatocellular carcinoma typically has a poor prognosis, because it is often diagnosed at an advanced stage (Ragagopal et al., 2008). Early diagnosis of HCC greatly improves its prognosis. However, the distinction between benign and malignant tumors is often difficult and novel immunohistochemical markers are necessary (Masanori et al., 2008). Expression of HSP70 increases under conditions of environmental cellular stress and overexpression of HSP70 also leads to significant protection against cell apoptosis (Amal et al., 2013). The expression of HSP70 has been routinely associated with cancer progression, therapy responses, and poor prognosis in multiple cancers and it can be used as a novel biomarker for early detection of cancers (Yang et al., 2012). Ki67 expression was found to be in close relation to tumor growth rate, which was the most widely used proliferation-associated marker (Cezar et al., 2008). Early detection with appropriate therapy is still the optimal approach that offers the patient the best prognosis (Aileen, 2011). Overexpression of endothelial cell marker CD34 with gradual progression was found from normal liver to cirrhosis to HCC and metastasis. Understanding of this process of angiogenesis might help in the design of efficient and safe anti-angiogenic therapy for these liver disorders (Amarapukar et al., 2008).

The aim of this work: to evaluate of expression of HSP70, CD34 and Ki 67 in liver cirrhosis and early detection of hepatocellular carcinoma.

**MATERIALS AND METHODS**

This work included 60 liver biopsies obtained from Al Azhar University hospitals as paraffin blocks. Four-micron thick sections were cut from paraffin blocks of all cases and stained with haematoxylin and eosin (H&E). All cases were re-evaluated and classified into 3 groups; Group 1: Control (10 biopsies), Group 2: Liver Cirrhosis (25 cases), and Group 3: HCC (25 cases). The primary antibodies were as follows:

**Heat Shock Protein 70 (HSP70)** monoclonal antibody SPA810, clone C92 (StressGen Biotechnologies) at a final concentration of 10 mg/ml. Biotinylated secondary antibody, an avidinbiotin complex (Vector Laboratories; Burlingame, CA), and 3,39-diaminobenzidine (Sigma Biochemicals; St Louis, MO) as a chromogen were applied for visualization of the immunoreactions. According to (Luca et al., 2007), HSP70 positivity (nuclear & cytoplasmic) evaluated as follows: + (5–10% reactive cells); ++ (11–50% reactive cells); +++ (>50% reactive cells).

**CD34** (Monoclonal Mouse Anti-Human Class II, code No. M7165) was used at a dilution range of 1:25-1:50. CD34 staining was scored into four grades according to (De Boer et al., 2000): Negative; (When no capillaries were stained except those in the portal tracts), Minimal; (when only a few scattered individual capillaries were stained away from the portal tracts), Focal; (When some grouped capillaries were stained away from the portal tracts), and Diffuse; (When there was strong positivity throughout the capillaries all over).

**Ki-67** (Monoclonal Mouse Anti-Human Antigen, Code M7240), was
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used at a dilution range of 1:75-1:150. According to Elena et al., (2012), Ki 67 positivity evaluated according to percentage of positive cells into four-degree: (-) < 24%, (+) 25-50 % (Isolated), (+++) 51-74% (Focal), (+++) > 75 % (Diffuse).

Statistical Analysis: Patients’ data were tabulated and processed using SPSS (10.0) statistical package for Windows XP. Qualitative data were expressed by frequency and percent and were analyzed using Chi-square.

RESULTS

This study consisted of 60 liver biopsies; 10 cases as a control and 50 cases of hepatic lesions

I. Immunohistochemical results:

A. HSP70: (Fig 1 and tables 1&2).

HSP70 was negative in 10 out of 10 control biopsies (100%). Low expressed (+) in 2 out of 25 cirrhotic biopsies (8%) (Fig 6), and positive in 25 out of 25 HCC biopsies (100%); low expressed (+) in 5 out of 5 well-differentiated HCC (20%), intermediate expressed (++) in 13 cases (11 out of 11 moderately differentiated HCC and 2 out of 9 poorly differentiated HCC (52%), and high expressed (+++) in 7 out of 9 poorly differentiated HCC (28%).(Fig 7).

Table 1: HSP70 Expression among studied groups

<table>
<thead>
<tr>
<th>Expression</th>
<th>Control</th>
<th>Cirrhosis</th>
<th>HCC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>Negative</td>
<td>10</td>
<td>100.00%</td>
<td>23</td>
</tr>
<tr>
<td>Low expression</td>
<td>0</td>
<td>0.00%</td>
<td>2</td>
</tr>
<tr>
<td>Intermediate expression</td>
<td>0</td>
<td>0.00%</td>
<td>0</td>
</tr>
<tr>
<td>High expression</td>
<td>0</td>
<td>0.00%</td>
<td>0</td>
</tr>
</tbody>
</table>

HSP70 was highly significant \(X^2 = 54.497; p = 0.000\) among studied groups

![Graph of HSP70 Expression](image)

**Fig. 1:** Comparison of HSP70 among studied groups
Table 2: HSP70 expression in relation to tumor grade

<table>
<thead>
<tr>
<th>Expression</th>
<th>Well Differentiated</th>
<th>Moderately Differentiated</th>
<th>Poorly Differentiated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>Low expression</td>
<td>5</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Intermediate expression</td>
<td>0</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>High expression</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

HSP70 was highly significant ($X^2 = 41.453; p$-value<0.01) with tumor grade

B. CD34: (Fig 2 and tables 3&4)

CD34 was negative in all control biopsies. In cirrhotic biopsies, expression was in the portal and periportal sinusoids of the nodules (Fig 8). It is minimal in 5 out of 25 (20%) and diffuses in 6 out of 25 (24%). It was positive in all cases of HCC (100%); focal in one case (4%) and diffuse in sinusoidal spaces throughout the lesion in 24 cases (96%) (Fig 9).

Table 3: CD34 Expression among studied groups

<table>
<thead>
<tr>
<th>Expression</th>
<th>Control</th>
<th>Cirrhosis</th>
<th>HCC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>Negative</td>
<td>10</td>
<td>100.00%</td>
<td>0</td>
</tr>
<tr>
<td>Minimal</td>
<td>0</td>
<td>0.00%</td>
<td>5</td>
</tr>
<tr>
<td>Focal</td>
<td>0</td>
<td>0.00%</td>
<td>0</td>
</tr>
<tr>
<td>Diffuse</td>
<td>0</td>
<td>0.00%</td>
<td>6</td>
</tr>
</tbody>
</table>

CD34 was highly significant ($X^2 = 92.480; p = 0.000$) among studied groups

Fig. 2: Comparison of CD34 among studied groups
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Table 4: CD34 expression in relation to tumor grade

<table>
<thead>
<tr>
<th>Expression</th>
<th>Well Differentiated</th>
<th>Moderately Differentiated</th>
<th>Poorly differentiated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>Focal</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Diffuse</td>
<td>5 100</td>
<td>11 100</td>
<td>8 88.9</td>
</tr>
</tbody>
</table>

CD34 was insignificant ($X^2 = 1.852; p-value = 0.398$) with tumor grade.

C. Ki 67: (Fig 3 and tables 5&6)

Ki67 was negative in all control biopsies, isolated positive in one cirrhotic case (4%), while in HCC, it was negative in 2 cases (8%) (Fig 10), isolated positive in 9 cases (36%), focal positive in 10 cases (40%) and diffusely positive in 4 cases (16%). (Fig 11).

Table 5: Ki 67 Expression among studied groups

<table>
<thead>
<tr>
<th>Expression</th>
<th>Control</th>
<th>Cirrhosis</th>
<th>HCC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>Negative</td>
<td>10 100.00%</td>
<td>23 92.00%</td>
<td>2   8.00%</td>
</tr>
<tr>
<td>Isolated positive</td>
<td>0 0.00%</td>
<td>1 4.00%</td>
<td>9   36.00%</td>
</tr>
<tr>
<td>Focal Positive</td>
<td>0 0.00%</td>
<td>0 0.00%</td>
<td>10 40.00%</td>
</tr>
<tr>
<td>Diffuse Positive</td>
<td>0 0.00%</td>
<td>1 4.00%</td>
<td>4   16.00%</td>
</tr>
</tbody>
</table>

Ki67 was highly significant ($X^2 = 45.531; p = 0.000$) among studied groups.

Fig. 3: Comparison of Ki 67 among studied groups

Table 6: Ki 67 expression in relation to tumor grade

<table>
<thead>
<tr>
<th>Expression</th>
<th>Well Differentiated</th>
<th>Moderately Differentiated</th>
<th>Poorly Differentiated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>Negative</td>
<td>2 40</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Isolated positive</td>
<td>3 60</td>
<td>6</td>
<td>54.5</td>
</tr>
<tr>
<td>Focal Positive</td>
<td>0 0</td>
<td>5</td>
<td>45.5</td>
</tr>
<tr>
<td>Diffuse Positive</td>
<td>0 0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
From the previous table it was shown that 60% of well differentiated tumors showed isolated positive expression, and moderately differentiated tumors ranged between isolated and focal positive expression (54.5% and 45.5% respectively), while poorly differentiated tumors ranged between focal and diffuse positive expression (55.6% and 44.4% respectively). On comparison of KI67 with tumor grade, it was found statistically highly significant ($X^2 = 22.828; p$-value<0.01).

II. Histopathological results:

Ten biopsies of the control group, 25 biopsies of liver cirrhosis (12; with no dysplastic changes (Fig 4), 9; with low-grade dysplasia, and 4; with high-grade dysplasia), and 25 biopsies of HCC (9; acinar type (Fig 5), 12; trabecular type, and 4; poorly differentiated type.
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Fig 4: Liver showing regenerative nodules surrounded by fibrous bands (H&E X 150)

Fig 5: HCC: malignant hepatocytes arranged in acinar pattern (H&E X 360)

Fig 6: Liver cirrhosis negative for HSP70 (Immunostain X 200)

Fig 7: HCC showing intermediate positivity for HSP70 (Immunostain X 400)

Fig 8: Cirrhosis showing CD34 positivity in the portal and periporal sinusoids (Immunostain X 200)

Fig 9: HCC showing diffuse positivity for CD34 throughout lesion (Immunostain X 200)

Fig 10: Cirrhosis negative for Ki 67 (Immunostain X 200)

Fig 11: HCC showing Ki 67 nuclear positivity (Immunostain X 200)
DISCUSSION

In this study, HSP70 was negative in all control biopsies (100%), low expression in 2 of 25 (4%) of cirrhotic biopsies, while it is positive in all biopsies of hepatocellular carcinoma as low expression in 5 cases (20%), intermediate expression in 13 biopsies (52%) and high expression in 7 biopsies (28%).

Our findings are in agreement with Shin et al., (2011) who found that HSP70 staining in 282 of 392 HCC samples (71.9%), and with (Luca et al., 2007) who found that HSP70 immunoreactivity was seen in the vast majority of HCCs (39 of 53 cases, 73.58%), including 9 of 10 cases of HCC (90%) and 16 of 22 (72.7%) for G1 HCC, and with Effendi & Sakamoto (2010) who detected immunoreactivity of HSP70 ranging up to 80% in most cases of early HCC, while no or only focal and faint nuclear staining was observed in the noncancerous background liver tissue, and with (Amal et al., 2013) who found that overexpression of HSP70 detected in 92.86% of early Hepatocellular carcinoma cases, while the expression was noticed in 2.94% of liver cirrhosis cases without hepatocellular carcinoma.

According to the Mee et al., (2005), HSP70 may play an important role in hepatocarcinogenesis, and can contribute tumor progression by promoting tumor cell proliferation in HCC, so its expression can contribute to not only hepatocarcinogenesis but also tumor.

Our findings are in agreement with (Di Tommaso et al., 2007) that use a panel of markers on HCC samples. Heat shock protein 70 (HSP70), glypican 3 (GPC-3), and glutamine synthetase (GS) were shown to offer a sensitivity of 70% and a specificity of 100% (for 2 markers) Even for very well differentiated and grade 1 HCC, the accuracy was 57% (3 markers) and 72.9% (2 markers) and a 100% specificity.

In our study, HSP70 was expressed in all HCC patients with different degrees of expression, although this comes in contrast with El-Din et al., (2010) who found that HSP70 was not associated with high risk of future development of HCC in Egyptian population. Our findings are in disagreement with Chuma et al., (2003) who reported HSP70 as the most abundantly up-regulated gene in early HCC and significantly over expressed in advanced HCC as compared to early HCC and precancerous lesions.

In this study, CD34 was observed as negative in all normal cases, while in cirrhotic cases, it expressed as minimal in 5 cases (20%) and diffuse in 6 cases (24%), whereas it is positive in all cases of hepatocellular carcinoma as focal in one case (4%) and diffuse in 24 cases (96%).

Our findings are in agreement with (Yang et al., 2010; Ding et al., 2011) who reported that CD34 staining is one of the important markers of tumor angiogenesis and microvascular density in HCC and is closely associated with HCC prognosis, and with Wang et al. (2012) who study liver thin-core biopsy specimens, and found that the sensitivity and specificity of CD34 expression in small HCC nodules (<3 cm) were 94% and 89%, respectively, and with (Wee A& Sampatanukul P, 2004) who reported that the positive rate of CD34 expression in HCC cases were 100%, and with Paschoal et al., (2014) who observed that, CD34 endothelium markers were positive in all types of hepatocellular nodules, although with variable intensity, and with Du et al., (2011) who found the positive expression rates of CD34 in advanced
HCC and well-differentiated HCC ranged from 25% to 100% [and strongly positive in 76% (38/50) and 70% (21/30), respectively]

CD34 expression in the capillaries and sinusoids of noncancerous hepatic tissue is a risk factor for multicentric recurrence of HCC and histologic assessment of hepatic tissue with CD34 immunohistochemistry might be useful for the prognostic evaluation of HCC patients after surgery (Tsuji et al., 2013).

In liver carcinogenesis, CD34 antibody is one of the most studied vascular markers. It is important for the prognostic evaluation of patients and also has diagnostic value (Yao et al., 2007).

In this study, Ki 67 staining was observed as negative in all normal cases, isolated positive in one cirrhotic case (4%) while in cases of hepatocellular carcinoma expressed as negative in 2 cases (8%), isolated positive in 9 cases (36%), focal expression in 10 cases (40%) and diffuse positive in 4 cases (16%).

Our findings are in agreement with (Mary et al., 2006) who found that, there was a statistically significant trend of increasing Ki67 expression from large regenerative nodule, dysplastic nodule to hepatocellular carcinoma, and with (Quaglia et al., 2006) who reported that Ki67 could represent a valuable tool in the understanding of HCC progression in cirrhosis.

**Conclusion**

We concluded that, there is a role of HSP70, CD34 and Ki-67 in differentiation of non-neoplastic liver cirrhotic lesions and early HCC and these markers can be used as predictors of biologic behavior of HCC. We recommended performing prospective studies with clinical correlation between these markers and the progression of HCC with large series of patients.

**REFERENCES**


De Boer M.B.B.S., W. Bastiaan M.B.B.S.*, Amanda Segal M.B.B.S., Felicity A Frost


Elena Mocanu1, V. Broască2, Beatrice Severin (2012): Ki-67 Expression In Hepatocellular Carcinoma Developed On A Liver Cirrhosis ARS Medica Tomitana 1;(68):


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