Expression of CD29 and CD49 in HCV-Patients with Chronic Hepatitis, Cirrhosis and Hepato Cellular Carcinoma.

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Abstract: The aim of this work is to study the expression of the integrins CD29(β1) and CD49 (α3)on hepatocytes in HCV patients with chronic hepatitis, cirrhosis, and HCC. Ninety patients (72 males and 18 females) were the subject of this study. Patients were admitted to the Department of Gastroenterology and Hepatology, Theodor Bilharz Research Institute, Giza, Egypt. They included 35 cases of chronic hepatitis C virus infection (CH), 25 cases with cirrhosis and 30 cases of HCC. Liver biopsy was done for histopathologic and immuno-histochemical (IHC) studies Ten normal control liver biopsies were from individuals subjected to laparoscopic cholecystectomy. Liver biopsy sections were evaluated for the histopathological and basic classification as chronic hepatitis and cirrhosis. Immunohistochemical reaction was performed using an avidin biotin complex (ABC) immunoperoxidase technique using anti human CD29, and CD49 on paraffin sections. No tissue CD29 expression was detected in the control group. There is statistical significant difference in tissue CD29 expression between the CH & LC groups relative to the control group $p < 0.05$. The increase in HCC group was statistically significant relative to the control group $p < 0.001$. Tissue CD29 expression in CH groups is statistically significantly less relative to the HCC group $p < 0.001$. Again, there was significant statistical difference between CH and LC $p < 0.001$.No tissue CD49 expression was detected in the control group. There was statistical significant difference in tissue CD49 expression between the CH & LC groups relative to the control group $p < 0.05$. While in HCC group, it was statistically significant relative to the control group at a $p < 0.001$. Tissue CD49 expression in CH groups is statistically significantly less relative to the HCC group $p < 0.001$. Tissue CD49expression in LC groups is statistically significantly higher relative to the CH group $p < 0.001$. In conclusion, CD29 and CD 49 integrins were highly expressed in HCC and cirrhotic patients and this may be related to fibrosis and consequently culminating into HCC. The expression of such integrins could explain the invasive and recurrent nature of HCC. Further researches in this field may open a novel approach for the management of HCC.

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1.Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide affecting 1 million individuals annually.(1) Intensive research efforts have been directed toward the identification of novel treatment strategies and markers associated with the initiation and progression of HCC.(2)

However, despite advances in the detection and treatment of the disease, mortality rate remains high because current therapies are limited by the advanced stage in which the disease is usually diagnosed, when most potentially curative therapies such as resection and transplantation are of limited efficacy. (3)

Furthermore, the emergence of chemotherapy-resistant cancer cells leaves this disease with no effective therapeutic options and a very poor prognosis.(3)

It is widely recognized that the alteration of the expression of cell surface components is accompanied by malignant transformation.(4)

CD29 (β1-integrin) and CD49F (α6-integrin) are cell adhesion molecules and are important for cell matrix interactions. These markers are also used to study early hepatocyte differentiation.(5)

Galactoproteins b are a group of cell membrane glycoproteins that show enhanced expression after oncogenic transformation of fibroblasts. (6) One of these glycoproteins, called galactoprotein b3, was later identified as integrin α3subunit (CD49c) through cDNA cloning. (7,8). It is physically associated with the integrin β1 subunit (CD29) to form the α3β1integrin heterodimer. The integrin family of adhesion molecules is a major class of adhesion receptors that mediate cell–cell and cell–extracellular matrix (ECM) protein interactions.

It was also reported that α3β1 integrin was abundantly expressed in hepatocellular carcinoma (HCC) primary and metastatic tissues, whereas its expression in normal and peritumoral liver parenchyma was observed at very low levels. (9)
The invasion of carcinoma cells is related to the interaction between cells and extracellular matrix (ECM) and it has been known that integrins are most important receptors mediating interaction between cells and ECM.(10)

Aim of this work:

The aim of this work is to study the expression of the integrins CD29 and CD49 on hepatocytes in HCV patients with chronic hepatitis, cirrhosis, and HCC.

2. Material and Methods:

Patients

Ninety patients (72 males and 18 females; mean age 40.3 ± 2.4, range 25-65 years) were the subject of this study. Patients were admitted to the Department of Gastroenterology and Hepatology, Theodor Bilharz Research Institute, Giza, Egypt. They included 35 cases of chronic hepatitis C virus infection (CH), 25 cases with cirrhosis and 30 cases of HCC. The presence of HCV-RNA in patients sera was detected by real-time polymerase chain reaction. They were subjected to thorough clinical examination, urine and stool analysis, liver function tests, ultrasonography and liver biopsy for histopathologic and immuno-histochemical (IHC) studies. The study protocol was approved by the Ethics Committee of TBRI according to the Institutional Committee for the Protection of Human Subjects and adopted by the 18th World Medical Assembly, Helsinki, Finland.

Ten control liver biopsies were from individuals subjected to laparoscopic cholecystectomy after their consent. They were 4 males and 6 females with a mean age of 48.3 ± 2.3 years. Their liver function tests were normal and had no serologic evidence of hepatitis B and/or C viruses.

Liver biopsies were fixed in 10% buffered formalin for 24 hours, and then processed in ascending grades of ethyl alcohol, xylene, wax and paraffin blocks. Sections (4 μm) were cut on albuminized glass slides and stained with Hematoxylin & eosin and Masson trichrome stains. All sections were subjected to light microscopic examination for evaluating the histopathological and basic classification as chronic hepatitis and cirrhosis according to Desm et al., (11) and Knodell *et al.,* (12), and HCC according to Edmondson H and Steiner (13)

They were evaluated on a five point scale, using 20 random fields at x100 and x400 magnification per slide. Architectural changed, fibrosis and cirrhosis were evaluated on a seven point scale according to Knodell score system (12). Other liver sections (4 μm) were cut on slides, which were treated with TESPA (3-aminopropyl-triethoxysilane, Sigma) for IHC.

Immunohistochemistry for Detection of CD29 and CD49 antigens:

Immunohistochemical reaction was performed using an avidin biotin complex (ABC) immunoperoxidase technique according to Hsu and Reine.(14) using anti human CD29, and CD49 on paraffin sections; dewaxed in xylene and hydrated in descending grades of ethanol. Endogenous peroxidase activity was quenched by incubation in 3% hydrogen peroxide in 100% methanol for 20 min. Antigen retrieval was performed by microwaving the sections in citrate buffer (pH 6.0) for 15 min at 700 W. Sections were incubated overnight at 4^{o}C with the anti-human primary antibodies against CD29 and CD49 (purchased from Santa Cruz Biotechnology Inc.; Santa Cruz, USA) monoclonal antibody, diluted at 1:150, 1:100 respectively in BPS. Next day, after thorough washing in PBS, the sections were incubated with streptavidin-biotin-peroxidase preformed complex and evidentiated using a peroxidase/DAB (diaminobenzidine) enzymatic reaction for CD29 & CD49 Staining is completed by 5-10 minutes incubation with 3, 3'-diaminobenzidine (DAB) + substrate - chromogen which results in a brown-colored precipitate at the antigen site for both (cytoplasmic stain). Slides were washed in PBS for 5 minutes. Slides were placed in 70%, 95% and then 100% alcohol each for 5 minutes. The cell nuclei were counterstained with Mayer's hematoxylin. The cover slips were mounted using Dpx.

Positive and negative control slides for each marker were included within each session. As a negative control, liver tissue section was processed in the above mentioned sequences but the omission of the primary antibody and PBS was replaced.

Evaluation of immunostaining:

Sections were examined under light microscopy at x 400 for CD29, CD49 Two features of the immunoreaction were assessed separately on a semi quantitative basis as, the mean percentage of the positively stained cells. The number of positively stained cells was recorded in ten microscopic fields with the highest expression and the percentage was calculated from their mean. Zero% was given to unstained sections. Pattern of all markers were cytoplasmic staining.

Statistical analysis:

The Statistical Package for Social Sciences (SPSS) for Windows (version 10) computer program was used for statistical analysis. For comparison of more than 3 group's means, one-way ANOVA test, Post Hoc test was used. Comparison between percent positive cases was calculated by Chi-square test. A *P* value < 0.05 was considered statistically significant.

3. Results:

Table (1): Tissue expression of integrins β1 CD29 in studied groups

Variable	CH (n=35) Mean ± SD	LC (n=25) Mean ± SD	HCC (n=30) Mean ± SD	Control (n=10) Mean ± SD
CD29	6.3±1.21 [(a)(c)(d)]	40. 3 ± 7.02 [(b)]	47.5 ± 13.2 [(b)]	0.0±0.0

[a]: *p* value <0.05 relative to the control group [b]: *p* value <0.001 relative to the control group
[c]: *p* value <0.001 relative to the HCC group [d]: *p* value <0.001 relative to the LC group

There is no tissue CD29 expression in the control groups whereas there is a high expression in the HCC group. There is statistical significant difference in tissue CD29 expression between the CH & LC groups relative to the control group as *p* value < 0.05. The increase in HCC group is statistically significant relative to the control group at a *p* value < 0.001. Tissue CD29 expression in CH groups is statistically significant relative to the HCC group as *p* value < 0.001. Again, there was significant statistical difference between CH and LC and *p* value is <0.001(Table 1, Figures 1&2)

Table (2): Tissue expression of α3 integrins CD49 In studied groups

Variable	CH (n=35) Mean ± SD	LC (n=25) Mean ± SD	HCC (n=30) Mean ± SD	Control (n=10) Mean ± SD
CD49	4.3±1.21 [(a)(c)(d)]	30. 3 ± 7.02 [(b)]	55.5 ± 11.2[(b)]	0.0±0.0

[a]: *p* value <0.05 relative to the control group [b]: *p* value <0.001 relative to the control group
[c]: *p* value <0.001 relative to the HCC group [d]: *p* value <0.001 relative to the LC group

There is no tissue CD49 expression in the control groups whereas there is a high expression in the HCC group. There is statistical significant difference in tissue CD49 expression between the CH & LC groups relative to the control group *p* value < 0.05. While in HCC group, it is statistically significant relative to the control group at a *p* value < 0.001. Tissue CD49expression in CH groups is statistically significant relative to the HCC group at a *p* value < 0.001. Tissue CD49expression in LC groups is statistically significantly higher relative to the CH group at a p value < 0.001(Table 2, Figures 3&4).

Fig.1:A case of CHC with cirrhosis, A2F2, showing moderate of CD29 as cytoplasmic stain in the hepatocytes (IHC, DAB, X200).

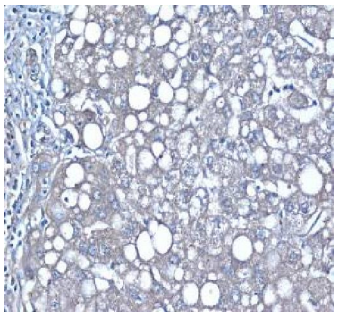


Fig.2:A case of moderately differentiated HCC, showing moderate to marked expression of CD29 in the cytoplasm of hepatocytes (arrow) (IHC, DAB, X200).

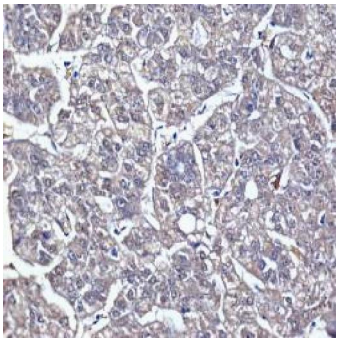


Fig.3:A case of CHC with cirrhosis, A2F2, showing mild to moderate of CD49 as cytoplasmic stain in the hepatocytes (IHC, DAB, X200).

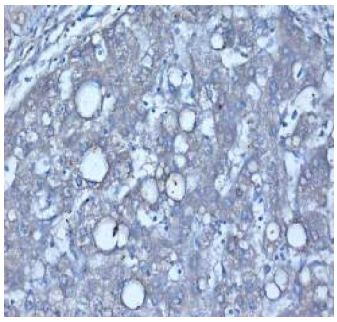
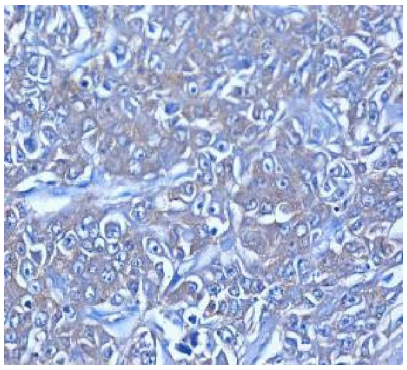


Fig.4:A case of moderately differentiated HCC, showing moderate to marked expression of CD49 in the cytoplasm of hepatocytes (arrow) (IHC, DAB, X200).

4.



Discussion:

HCC is the most common malignant tumor in the liver, and the prognosis of patients with this type of cancer is primarily determined by the incidence of recurrence after surgery and the occurrence of invading metastases into the remaining liver parenchyma. The formation of metastatic nodules of HCC involves an intricate multi-process cascade including cell adhesion, migration, and proteolysis of ECM. (15,16)

Patriarca *et al.* (1993) investigated the expression of **β1** integrin and collagen IV immunocytochemically and their data emphasized the neoplastic progression of HCC may be correlated with an aberrant expression of adhesion molecules/ and with a disruption of the collagen IV complement of basal membranes.(17)

Jaskiewicz &Chasen (1995), found relatively high activity of α3 and β1 integrins in metastatic tumors and the presence of all integrins in cirrhotic liver.(18)

Masumato *et al.* (1999) studied the activity of β1 integrins evaluated by cell adhesion to collagen, fibronectin and laminin, and found that the levels of constitutive activity of β1 integrins correlated with the invasive ability of HCC cells. Their results not only show an essential role of β1 integrins in invasion of HCC cells but also suggest subtle regulatory mechanisms of cell invasion.(19)

Giannelli *et al.* (2003) showed that expression of α3β1 was shown to be essential for HCC cell invasion into three-dimensional ECM structures. The migration of HCC cells on a laminin-5- coated plate was previously found to positively correlate with the level of expression of α3β1 integrin on the surface of these cells. These findings have suggested that the increased expression of α3β1 integrin on the surface of HCC cells is implicated in their malignant behavior.(20)

In our study, we found high expression of CD29 and CD 49 in HCC patients that was higher than CL and cirrhotic patients denoting their possible role in the progression of HCC. Nevertheless, their expression could be related to the invasive nature in our HCC patients.

Zhao *et al.* (2010) studied the expression of integrin β1 in HCC, hepatic cirrhosis normal liver tissues by (RT-PCR) and found that the level of integrin β1 mRNA in HCC and cirrhosis were much higher than that in the normal hepatic tissue. They concluded that integrin β1 may play an important role in the development of hepatic fibrosis, hepatic cirrhosis and hepatocellular carcinoma(21)

This study is matching with what we found that expression of β1 was higher in our HCC and cirrhotic patients.

Giannelli and co-workers(2002) stated that laminin-5 is present in the HCC primary nodule but not in the normal or cirrhotic peritumoral liver.(22) They also demonstrated a strong correlation between the expression of laminin-5 in HCC and the occurrence of metastasis. It is most likely that the interaction between α3β1 integrin on the HCC cell surface and laminin-5 in the surrounding tissues is critical for the invasion and metastasis of HCC.

Katabami *et al.* (2005) studied the human HCC cell line HepG2, and their studies showed the same findings of Gianneli et al.(23)

Reports have shown that transforming growth factor (TGF)-β1 stimulated non invasive HCC cells to transform into invasive cells in association with the enhanced expression of α3 integrin.(24) and that the upregulation of this integrin induced by TGF-β1 is mediated by the Ets-family of transcription factors.(23) The concentration of TGF-β1 in the serum of HCC patients has been shown to strongly correlate with the levels of expression of α3 integrin in the tumor tissues of these patients. (24)

In our chronic HCV, (TGF)-β1 may be responsible for expression of α3 integrin in our cirrhotic and HCC patients.

Recent efforts in stem cell biology suggest that tumors are organized in a hierarchy of heterogeneous cell populations and that the capability to maintain tumor formation/growth specifically resides in a small population of cells called *cancer stem cells* (CSCs).(3) These cancer stem cells were shown to express α3β1integrin (25)

However, the recent "cancer stem cell hypothesis" proposes the involvement of a minor population of cells with self-renewal capability in the pathogenesis of a variety of cancers, including HCC(26).

In terms of hepato-carcinogenesis, it has traditionally been believed that long-term, repeated injury and regeneration of damaged mature cells induce an accumulation of multiple genetic or epigenetic alterations, which ultimately lead to cancer.(27,28)

Moreover, it is considered that the development of tumors, at least in some cases of HCC, can be attributed to the propagation of the stem/progenitor cell component in hepato-carcinogenesis.(29)

Given the close association between inflammation and carcinogenesis, it is reasonable that chronic and persistent tissue injury such as hepatitis viral infection might expand and activate the stem cell pool, thus predisposing the patient to the initiation of cancer.(30)

Signaling pathways that regulate the self-renewal of stem cells are important drivers of cell proliferation and survival and are frequently relevant to carcinogenesis
when disrupted by mutations. (31,32)

Notably, both increased expression of the *Bmi1* gene and activation of the Wnt/_-catenin pathway are frequently observed in liver cancers such as hepatocellular carcinoma (HCC).(33,34)
These observations imply that the dysregulated self-renewal of hepatic stem/progenitor cells serves as an early event in hepatocarcinogenesis. (25)

Therefore, this study and others mentioned studies may open the way for new areas of research in understanding the pathogenesis of HCC as regards e.g. the role of laminin-5 and its relation to α3β1integrin, the role of (TGF)-β1 in pathogenesis and invasiveness of HCC.

Nevertheless, if there possible therapeautic implication in targeting α3β1integrin, laminin-5, and/or of (TGF)-β1.

Again, in the new era of stem cell biology, the CSCs with their unique markers could be target for therapy in the future.

Conclusion:

In conclusion, CD29 and CD 49 integrins were highly expressed in HCC and cirrhotic patients and this may be related to fibrosis and consequently culminating into HCC. The expression of such integrins could explain the invasive and recurrent nature of HCC. Further researches in this field may open a novel approach for the management of HCC.

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