Bull. Pharm. Sci., Assiut University, Vol. 34, Part 2, December 2011, pp. 181-187.

# THE EXPRESSION PATTERN OF GALECTIN-3 IN THE RAT HEPATOCELLULAR CARCINOMA

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يعتبر سرطان الكبد من الامراض شائعة الانتشار ولقد صنف هذا المرض فى المرتبة الثالثة كمسبب للوفاة على مستوى العالم ، ويبلغ عدد الوفيات واحد مليون تقريبا سنويا. ويعتير الجالكتين ٣ الاكثر انتشارا بين انواع مختلفة من الخلايا والانسجة، حيث وجد انه يؤثر على العديد من العمليات البيولوجية، وايضا له تاثير فى مختلف الظروف المرضية والفسيولوجية مثل النمو وردود الافعال المناعية والتحول السرطانى وانتشار السرطان فى الخلايا.

وفى هذه الدراسة وجد ارتفاع مستوى الجالكتين ٣ فى كلا من (البروتين والحامض النووى الرسول) فى خلايا الكبد المسرطنة مقارنة بخلايا الكبد الطبيعية، وبالاضافة الى هذا لاحظنا وجود الجالكتين ٣ بكمية ملحوظة فى نواة الخلايا السرطانية وان هذه الزيادة مصحوبة بزيادة فى انزيمات الكبد والنيتريت.

*Hepatocellular carcinoma (HCC) is the third most frequent cause of cancer death world wide, with an estimated mortality rate of about one million deaths annually. Galectin-3 is widely spread among different types of cells and tissues. Galectin-3 found intracellularly in nucleus and cytoplasm. Galectin-3 affects numerous biological processes and seems to be involved in different physiological and pathophysiological conditions, such as development, immune reactions, and neoplastic transformation and metastasis. In this study, we found over expression of galectin-3 protein and mRNA, in HCC tissue compared with normal liver tissue. In addition, we found expression of galectin-3 in the nuclear protein fraction. The overexpression of galectin-3 was accompanied with elevated levels of serum nitrite and aminotransferases (AST and ALT).*

## INTRODUCTION

Hepatocellular carcinoma (HCC) accounts for 85% to 90% of all primary liver malignancies. It is one of the most common cancers and the third leading cause of cancer mortality worldwide[1]. The development of HCC is related to chronic liver inflammation that leads to fibrosis and cirrhosis. However, HCC can be seen in non-cirrhotic patients with chronic viral hepatitis, toxin exposure, or other chronic liver diseases such as hemochromatosis[2].

Galectin-3, a 31-kDa unique trimeric gene product, is an intracellular and extracellular lectin which is presumed to interact with glycoproteins of the cell surface matrix. It consists of three structural domains: (a) an $NH_2$-terminal domain; (b) a repeated collagen-like sequence which is rich in glycine, proline, and tyrosine; and (c) a COOH-terminal carbohydrate recognition[3]. Galectin-3 is expressed widely in cancer cells as well as epithelial and immune cells[4&5]. Galectin-3 is shown to be involved in cell growth, cell proliferation, cell differentiation, cell adhesion, angiogenesis, apoptosis, and tumor progression and metastasis mainly through binding to glycoproteins. The expression of galectin-3 in tumor cells is correlated with tumor invasion and metastatic potential of several types of cancer[6]. Clinical evidences have shown that the expression of galectin-3 is associated with the carcinogenesis and malignant potential in melanoma, head and neck, thyroid, gastric, colon, uterine, and renal cancers[7&8].

Although galectin-3 is predominantly localized in the cytoplasm of several types of tumor cells, it has also been detected in the nucleus, suggesting that galectin-3 is a shuttling protein between the nuclear and cytoplasm cellular compartments and

Received in 17/11/2011 & Accepted in 12/12/2011

accordingly may have multiple functions[9]. In this study, we aimed to investigate the expression pattern of galectin-3 in hepatocellular carcinoma in a rat model.

## MATERIAL AND METHODS

### Chemicals

RNA extraction kit from (BioFlux, Bioer Technology Co., Ltd.). RT using RevertAid™ First strand cDNA synthesis kit (Fermentas). PCR using Tag master/high yield (Jena Bioscience). DNA ladder using low range DNA ladder 50-1kbp linear scale (Jena Bioscience). Protein marker using page Ruler™ prestained protein ladder (Fermentas). PVDF membrane (Amersham Hybond™-P GE Healthcare) was used. All the other chemicals and solvents used in the study were of analytical grade and were obtained either from Sigma Chemical Company or commercial suppliers, unless otherwise mentioned.

### Treatment and sampling

Male Albino rats (n= 20) (150±25 g body wt) were purchased from animal house of Assiut University. Rats were divided into two groups; first group (control group n= 5) received normal saline. Second group (n= 15) received diethylnitosamine (DEN) 200 mg/kg intraperitoneal. Five rats were sacrificed after 16 weeks. Unfortunately, two rats were died and the remaining eight survived rats were sacrificed after 20 weeks. Blood samples were collected and livers were excised rapidly and used for RNA preparation or homogenization in 20 mM Tris, 100 mM NaCl, 1 mM EDTA and 0.5% Triton X100 buffer. Protein content of liver homogenate was determined using Biuret reagent and bovine serum albumin (BSA) as standard. The protease inhibitors mix was added. Samples were aliquotted and stored at -80°C tell use.

### Histopathology

Formalin-fixed liver specimens were transferred to 70% ethanol and embedded in paraffin. Tissue sections (5 µm) were stained with haematoxylin and eosin (HE). At least three slides were prepared from each specimen and examined under Optica B-82 microscope for detection of pathological changes.

### Assessment of serum liver function tests and serum nitrite

Blood samples were collected by cardiac puncturing method, centrifuged and sera were isolated for serological studies. Serum ALT, AST and nitrite were estimated by using commercially available kits according to the manufacturers instructions (Biodiagnostic, Egypt).

### Subcellular fractionation

One gram of fresh liver was washed with ice cold PBS and homogenized in a Dounce homogenizer with 9 volumes of 0.25 M sucrose containing 2 µg/ml each of leupeptin and pepstatin-A and 0.1 mM PMSF. The homogenate was centrifuged at 800 xg for 10 min. The supernatant is the cytosolic fraction and the pellet is the nuclear fraction. The pellet was washed to remove the contaminant from the cytosol then re-suspended in appropriate volume of the homogenization. The protein content in all fractions was determined, denatured and stored at -70°C until use.

### Oligonucleotides used for amplifications

NCBI reference sequence: NM_031832.1 and NM_031144.2 were used for design primers for rat Gal-3, and β-actin respectively. The coding sequences were used to design the primer pairs and the distance between the two primers was 407 bases for the Gal-3 and 500 bases for the β-actin.

The primer sets as following:

Gal-3 upper: 5′-GGC AGA CGG CTT CTC ACT T-3′
Gal-3 lower: 5′-GGG CAT ATC GTA GGG CAC T-3′
β-actin upper: 5′-**CAT** GGA TGA CGA TAT CGC TG-3′
β-actin lower: 5′-CAT AGA TGG GCA CAG TGT GG-3′

### RNA preparation and RT-PCR

Total RNA fractions were prepared using total RNA extraction kit from BioFlux, Bioer Technology Co., Ltd. according to the instruction manual. The first strand cDNA was synthesized according to the instruction manual of RevertAid™ First strand cDNA synthesis kit (Fermentas) from rat liver total RNA. The PCR was performed using Tag master/high yield (Jena Bioscience) as the following condition: pre-denaturing for 5 min at 94°C then denaturing at 94°C for 30sec, annealing at 55°C, and extension at 72°C for one minute. The amplification was carried out in 28 cycles using Biometra cycler (Germany).

**SDS-PAGE and Western blotting**

50 µg from each protein homogenate were denatured by boiling for 5min in 2% SDS and 5% 2-mercaptoethanol and loaded in each lane. SDS–PAGE was done at 100 volts for 2 hrs using 12% gel. The electro-transfer was done using T-77 ECL semidry transfer unit (Amersham Biosciences) for 2 hrs. The membrane was blocked in TBS buffer that contains 0.05 Tween and 5% non-fat milk for one hour. The primary antibodies that used were rabbit polyclonal anti-galectin-3 and β-actin (SANTA CRUZ Biotechnology, INC). Polyclonal goat anti-rabbit immunoglobulin, conjugated to alkaline phosphatase (Sigma–Aldrich, Schelldorf, Germany), diluted 1:5000 served as a secondary antibody.

## RESULTS AND DISCUSSION

Hepatocellular carcinoma is one of the world's deadliest cancers, ranking third among all cancer-related mortalities. The liver, unique in its capacity for regeneration following injury, also gives rise to this malignancy commonly associated with the inflammatory state of advanced fibrosis, or cirrhosis[10]. Several hepatotoxic agents have been used in the induction of HCC in animal model. One of these agents is Diethylnitrosamine (DEN); DEN induces pericentral foci of small dysplastic hepatocytes and acts by ethylating nucleophilic sites in DNA[11], causing cirrhosis and multifocal HCC within 18 weeks[12].

Galectins are members of a growing family of animal lectins of which galectin-3 is the most extensively studied[13]. This lectin is composed of 2 domains: a carboxyl terminal domain that contains the carbohydrate-binding region and an amino-terminal domain consisting primarily of tandem repeats of 9 amino acids[14].

**Serum levels of aminotransferases**

A significant ($p < 0.01$) increase in rat serum AST and ALT levels in all time points in comparison to the controls. The mean ± SD were obtained from each group are shown in table 1. The mean AST activity levels were 19±3.3, 102.1±14.7, and 120.1±11.9 u/L (for control, 16 and 20 weeks) groups respectively. The ALT activity levels were 18±7.9, 81.5±12.5 and 90.3±12.03 u/L for control, 16 and 20 weeks respectively. The AST: ALT ratio was 1.25 for the 16 weeks group and 1.33 for the 20 weeks group in comparison to 1.05 for the control group

**Table 1:** Serum levels of AST, ALT (U/L) and their ratio in control, and in the hepatocellular carcinoma groups.

| | AST (U/L) | ALT (U/L) | AST / ALT |
|---|---|---|---|
| Controls (n= 5) | 19±3.3 | 18±7.9 | 1.055 |
| 16 weeks (n= 5) | 102.1±14.7** | 81.5±12.5** | 1.25 |
| 20 weeks (n= 8) | 120.1±11.9** | 90.3±12.03** | 1.33 |

Values are means ± SD.
P values are shown as *P< 0.05 **P<0.01 vs Control

**Serum nitrite levels**

Table 2 shows a significant increase ($p < 0.05$ and $p < 0.01$) in rats mean serum levels of nitrite at 16 and 20 weeks respectively after receiving DEN in comparison to the control group. The mean level order was 1.27±0.58, 2.3±0.64 and 3.3±0.7 µM (for control, 16 and 20 weeks respectively).

**Table 2:** Serum level of nitrite (µM) in control, and in the hepatocellular carcinoma groups.

| | Nitrite (µM) |
|---|---|
| Controls (n= 5) | 1.26 ± 0.58 |
| 16 weeks (n= 5) | 2.3 ± 0.45* |
| 20 weeks (n= 8) | 3.3 ± 0.73** |

Values are means ± SD.
*P< 0.05 **P< 0.01 vs Control

**Histopathological study**

To asses the changes in parenchymal cells of the liver after DEN administration, HE stained slides were examined 16 and 20 weeks after DEN administration, as shown in figure 1. Administration of DEN induced morphological deformations in the liver pronounced with chronic hepatitis with hydropic degeneration and macroregenerative nodules. These nodules show focal prominence of bile ductular proliferation and after 20 weeks of DEN

administration, severe morphological and histopathological deformations in the liver pronounced by focal large cell changes in a trabecular hepatocellular carcinoma were observed (Fig. 1).

**Galectin-3 expression in hepatocellular carcinoma**

The results exhibited over expression of Gal-3 protein synthesized by liver cells at all time points in all rats. Figure 2A shows the expression levels of Gal-3 protein in total liver homogenate for five rats from the control, 16 and 20 weeks groups. Figure 2B shows expression of Gal-3 protein in total protein homogenates (T), cytosolic fractions (C) and nuclear fractions (N) for a representative rat from each group.

**Galectin-3 mRNA expression**

The over expression of Gal-3 was found to be associated with the over expression of mRNA in all rats as shown in figure 3 and the highest expression was found after 20 weeks.

In the current study, we found over expression of galectin-3 protein as indicated by the very intense bands in both 16 and 20 weeks groups after DEN injection in the total homogenate. These results are in agreement with another model of cancer made by Hsu *et al*.[15], who found that: Firstly, up-regulation of galectin-3 expression in proliferating fibroblasts with pronounced nuclear localization. Secondly, while present in low or undetectable levels in certain normal cells, galectin-3 was highly expressed when these cells were neoplastically transformed.

The mechanism by which galectin-3 affects cell morphology and growth properties through the interaction with certain intracellular proteins was explained by Hsu *et al*.[15]. Galectin-3 has been shown to have a role in pre-mRNA splicing. In addition, while galectin-3 is diffusely present in the cytoplasm, it becomes localized in the nuclei of proliferating cells, suggesting that this protein may be involved in the regulation of cell growth.

The expression pattern of galectin-3 in both the cytosolic and nuclear fractions is referred to what is known as "nucleocytoplasmic shuttling" that was defined by Borer *et al*.[16], as the repeated bidirectional movement of protein across the nuclear membrane. Also, Nakahara *et al*.[17], reported that the shuttling is very significant as it might be developed as a therapeutic modality to inhibit and/or regulate gene expression related to cancer. The mechanism of nuclear import of galectin-3 is explained by Gong *et al*.[3], who reported that the $NH_2$-terminal region of Gal-3 is responsible for its nuclear localization, whereas Gaudin *et al.*[18] have argued that, and stated that the last 10 amino acids of the COOH-terminal region of Gal-3 are the responsible terminus for its nuclear transport properties[19].

Many authors clarified the role of expression pattern of galectin-3 in different tumor cells where; cytoplasmic galectin-3 has an important role in its anti-apoptotic activity but has an opposite effect when localized in the nucleus[20]. In human prostate cancer, over-expression of galectin-3 in the cytoplasm can promote its anti-apoptotic activity as well as increase cell proliferation, tumor growth, invasion, and angiogenesis while galectin-3 expression in the nucleus decreases cell proliferation[20].

Serum AST and ALT activity levels have been used to evaluate the extent of the damage in the liver, and their ratio (AST/ALT) is another good indicator of the state of the liver and our results are in agreement with Sherman *et al*.[21].

Concerning nitrite-mean levels, the present study results are in agreement with Moriyama *et al*.[22], who found that the plasma concentrations of nitrite increased in patients with HCC are correlated with tumor volume. The role of nitrite in tumoristatic and tumoricidal effect through the mitochondrial injury of tumor cells was explained by Brown *et al*.[23], Kupffer cells, the largest population of fixed tissue macrophages, form an important component of the immune system in the liver[23]. It has been reported that the cytotoxicity of Kupffer cells against hepatoma cells is related to NO[24].

In conclusion Galectin-3 might be a potential therapeutic target for the prevention and treatment of hepatocellular carcinoma. Moreover, the nucleo/cytoplasmic shuttling of gal-3 might play a role in inhibition and/or regulation of gene expression related to cancer.

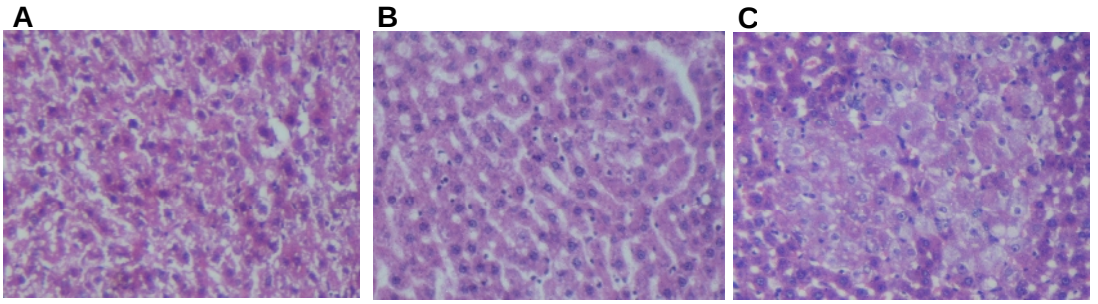


**Fig. 1:** Histopathological changes in hepatocellular carcinoma.

(A) Normal histology of liver with polygonal hepatocytes with prominent nucleus and maintained sinusoidal space B) 16 weeks after DEN administration induced morphological deformations in the liver pronounced with chronic hepatitis with hydropic degeneration and macroregenerative nodule. This nodule shows a focal prominence of bile ductular proliferation. C) 20 weeks after DEN administration induced severe morphological and histopathological deformations in the liver pronounced by focal large cell change in a trabecular hepatocellular carcinoma. Some tumor cells contain intranuclear cytoplasmic inclusions (HE, 400×).

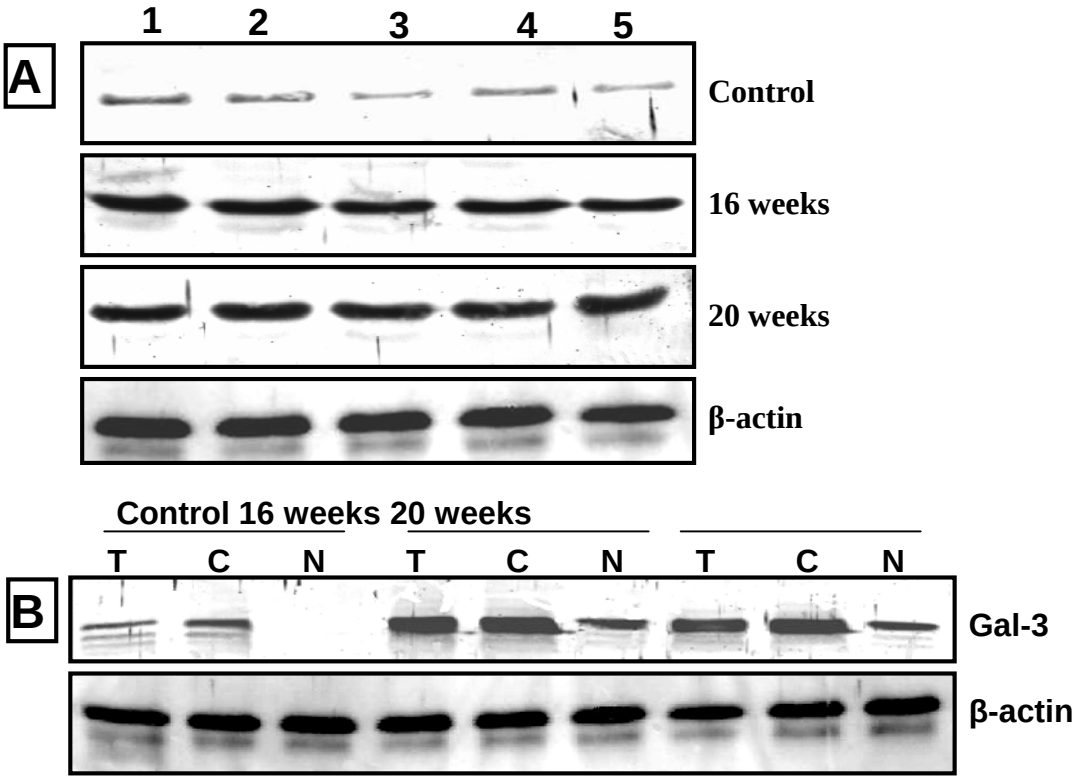


**Fig. 2.** Galectin-3 expression in hepatocellular carcinoma.

A) Galectin-3 expression of 50 µg of total liver homogenate were used from control group (n= 5 rats), 16 weeks group, and 20 weeks group. B) 50 µg of total liver homogenate (T), cytosolic fraction (C) and nuclear fraction (N) were used from control group, 16 weeks group, and 20 weeks group. Rabbit polyclonal antibodies for Gal-3 were used in 1:200 dilution. Anti-rabbit secondary antibody conjugated to alkaline phospatase was used in dilution 1:3000. Data are representative of three separate experiments. β-actin re-probed on the same immunoblot to sure the identity of loading.

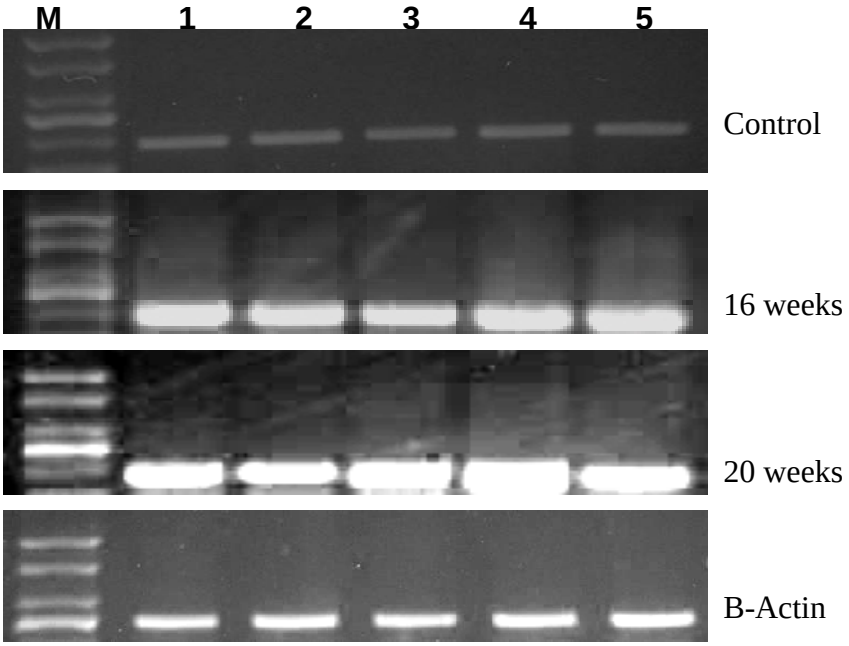


**Fig. 3:** RT-PCR of Gal-3 expression.

mRNA expression of Gal-3 in the control and after 16 weeks and 20 weeks of receiving DEN. B-actin used as internal control gene to show the integrity of the RNA. M: DNA marker and 1-5 represent five rats from each group.

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