Molecular and cellular pharmacology

Evaluation of antiglypican-3 therapy as a promising target for amelioration of hepatic tissue damage in hepatocellular carcinoma

Randa A. Zaghloul, Mamdouh M. El-Shishtawy, Khaled H. Abd El Galil, Mohamed A. Ebrahim, AbdelHamid A. Metwaly, Mohammed M. Al-Gayyar

ABSTRACT

In Egypt, hepatocellular carcinoma (HCC) was predicted to continue to rise over the next few decades causing a national problem. Meanwhile, glypican-3 (GPC3), a highly expressed glypican, has emerged as a potential target for HCC immunotherapy. Therefore, we aimed to identify the impact of blocking GPC3 on liver damage in HCC as well as a possible mechanism. Fifty four HCC patients, 20 cirrhotic patients and 10 healthy subjects were recruited. Serum levels of GPC3, sulfatase-2 (SULF-2), heparan sulfate proteoglycan (HSPG), insulin-like growth factor-II (IGF-II) were measured by ELISA. In parallel, HCC was induced in 40 male Sprague-Dawley rats in presence/absence of antiGPC-3. Liver impairment was detected by investigating liver sections stained with hematoxylin/eosin and serum α-fetoprotein (AFP). Liver homogenates of GPC3, SULF-2, and HSPG were measured by ELISA. Gene expression of caspase-3 and IGF-II were assayed by RT-PCR. HCC patients showed significant elevated serum levels of GPC3, IGF-II and SULF-2 accompanied by decreased HSPG. However, treatment of HCC rats with antiGPC-3 significantly reduced serum AFP and showed nearly normal hepatocytes. In addition, antiGPC-3 significantly reduced elevated liver homogenates protein levels of GPC3 and SULF-2 and gene expression of IGF-II and caspase-3. antiGPC-3 restored the reduced hepatic HSPG. antiGPC-3 showed anti-tumor activity as well as hepatoprotective effects. antiGPC-3-chemothapeutic effect can be explained by forced reduction of IGF-II expression, restoration of HSPGs, deactivation of SULF-2 and reduction of gene expression of caspase-3. Targeting GPC3 is a promising therapeutic approach for HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common type of cancer and the third leading cause of cancer-related death (Majumdar et al., 2012). The incidence of HCC varies throughout the world, with rising incidence in Egypt (Lehman and Wilson, 2009). Several therapeutic options have emerged as a possible treatment or management of HCC. However, hepatectomy offers the best outcomes for patients with HCC (Cherqui et al., 2009). But even with surgery as an optimum procedure for HCC, there are less than 30% of cases are amenable to hepatectomy at the time of diagnosis due to advanced tumor stage and underlying liver cirrhosis (Llovet and Bruix, 2008). Immunotherapy is a potentially attractive option for treating HCC, and the induction of tumor-specific reactions without autoimmunity is the ideal strategy (Nobuoka et al., 2013).

Glypicans (GPCs) are members of glypican family of glycosylphosphatidylinositol-(GPI)-anchored cell-surface heparan sulfate proteoglycans (HSPG) (Chen et al., 2014; Fimnus et al., 1995; Fu et al., 2013). Glypicans are generally and predominantly expressed during development. Their expression levels change in a stage- and tissue-specific manner, suggesting an involvement in morphogenesis.
GPC3 is highly expressed in HCC tissues where it has been proved to stimulate in vitro and in vivo growth of HCC and not in normal tissue (Capurro et al., 2005b; Hippo et al., 2004; Nakatsuura et al., 2003; Nishimura et al., 2008; Zhu et al., 2001). GPC3 has been demonstrated to interact with growth factors, such as insulin-like growth factor (IGF)-II and its receptor, with subsequent activation of its signaling pathway (Cheng et al., 2008).

IGF-II is a polypeptide of 67 amino acids (Daughaday and Rotwein, 1989) structurally homologous to insulin, insulin-like growth factor I and relaxin (Yao et al., 2007). IGF-II mRNA expression was detected in hepatocytes as well as in non-parenchymal liver cells such as Kupffer, endothelial, and hepatic stellate cells. It is highly expressed during hepatocarcinogenesis (Yang and Rogler, 1991). Activation of the IGF signaling pathway seems to play a role in the proliferation of a variety of cell types, such as fibroblasts, smooth muscle cells, chondrocytes, hemopoietic cells and others (Pietrzkowski et al., 1993) as well as the initiation and maintenance of oncogenesis (Nardone et al., 1996; Resnicoff et al., 1995). Only few studies have shown the physical interaction of GPC3 and IGF-II (Cheng et al., 2008; Pilia et al., 1996). GPC3 is hypothesized to confer oncogenecity through the interaction between IGF-II and its receptor, and the subsequent activation of IGF-signaling pathway (Cheng et al., 2008).

Sulfatase-2 (SULF-2) is an enzyme with 6-O desulfatase activity on heparan sulfate glycosaminoglycan (HSGAG) chains of GPC3 and other HSPG. SULF-2 is upregulated in 60% of HCC patients and associated with a worse prognosis (Lai et al., 2010). It acts as an oncoprotein in HCC development by promoting tumor growth by releasing growth factors from HSGAG storage sites at the cell surface and in the extracellular matrix, thus increasing the local concentration of growth factors available to bind to cell surface receptors and enhancing cell signaling (Lai et al., 2010). The interaction between SULF-2 and GPC3 revealed that forced expression of SULF-2 enhanced GPC3 expression both in vitro and in vivo (Chen et al., 2014). Therefore, the following study was conducted to evaluate the chemopreventive and hepatoprotective effects of antiGPC-3 in an in vivo model of HCC.

## 2. Materials and methods

### 2.1. Patients

From April to November, 2013, 54 patients with HCC (10 females and 44 males; aged 28–80 with a mean ± S.E.M. of 57.6 ± 1.39 years) were recruited from the Oncology Center, Mansoura University, Mansoura, Egypt. To nullify the effect of cirrhosis as underlying disease, since all HCC patients selected were diagnosed with HCC passing the cirrhotic stage, a group of 20 cirrhotic patients (5 females and 15 males; aged 38–59 years with a mean ± S.E.M. of 53.7 ± 1.76), without any evidence of HCC, were selected from the out-patient clinics of Specialized Medical Hospital, Mansoura University, Mansoura, Egypt. The study was approved by the local institutional ethical committee and patients’ consents were obtained according to the regulations of the Egyptian Ministry of Health. Patients’ characteristics were summarized in Table 1. All involved cases, HCC and cirrhotic patients, were diagnosed by clinical, radiological and pathological examination in the Oncology Center and the Specialized Medical Hospital, Mansoura University including; abdominal ultrasonography and serum AFP, with or without triphasic CT scan and/or liver histopathology. Severity of liver disease was assessed by Child-Pugh classification (Pugh et al., 1973). The stage and management were defined according to Barcelona-Clinic Liver Cancer (BCLC) group diagnostic and

### Table 1

Patients characteristics.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Male</th>
<th>Female</th>
<th>No</th>
<th>Yes</th>
<th>HCV</th>
<th>HBV</th>
<th>Both HCV and HBV(+)</th>
<th>N/A</th>
<th>HCC (n=54)</th>
<th>Cirrhosis (n=20)</th>
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<td>9</td>
<td>10</td>
<td>11</td>
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<td>6</td>
<td>4</td>
<td>14</td>
<td>9</td>
<td>3</td>
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<td>Etiology of liver disease</td>
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<td>13</td>
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<td>4</td>
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<td>11</td>
<td>9</td>
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</table>

<sup>a</sup> N/A: not available.
<sup>b</sup> BCLC: Barcelona-Clinic Liver Cancer.

### Table 2

The primer sets used for the detection of rat GAPDH<sup>4</sup>, IGF-II<sup>5</sup> and Caspase-3.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Reference sequence</th>
<th>Amplicon size</th>
<th>Annealing temperature (°C)</th>
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<td>NM_017008.3</td>
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<td>Rat GAPDH 358R</td>
<td>5'-CCAGACATCAGCAAGGAC-3'</td>
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<td>Rat IGF 1911F</td>
<td>5'-AGAGGCTTCAAGGAGAACACT-3'</td>
<td>NM_031511.2 NM_001190162.1 NM_001190163.1</td>
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<td>Rat Caspase 2111F</td>
<td>5'-CGCTCGTGTCCAGAGCTTC-3'</td>
<td>NM_012922.2</td>
<td>115</td>
<td>60</td>
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<tr>
<td>Rat Caspase 2296R</td>
<td>5'-ATCGTAGGCCCCTCTTCCTGT-3'</td>
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</table>

<sup>4</sup> GAPDH: glyceraldehyde-3-phosphate dehydrogenase.
<sup>5</sup> IGF-II: insulin-like growth factor.
treatment strategy (Llovet et al., 2004). Patients with other types of malignancy, advanced organ failure, active infection and advanced medical co-morbidity were excluded from the study.

Control group: It consisted of 10 healthy subjects (4 females and 6 males; aged 30–56 years with a mean ± S.E.M. of 47.8 ± 2.7 years) with no apparent evidence of active disease or medical disorder.

Blood sampling: 5 ml of blood was collected by vein puncture from fasted patients and control subjects, and then centrifuged at 4000 revolution per min (rpm) (Sigma centrifuge 2-16P, USA) for 5 min to obtain clear non-hemolysed sera. If analysis was not performed immediately, the sera were maintained at −80 °C until use.

2.2. Animals

Animal protocol was approved by the ethical committee of the Faculty of Pharmacy, Mansoura University, Mansoura, Egypt. Forty Sprague Dawley adult male rats weighing 180–200 g were purchased from the Vaccine and Immunization Authority (Helwan, Cairo, Egypt) and housed (Animal house, Faculty of Pharmacy, Mansoura University, Mansoura, Egypt). Rats were kept for the first week to allow acclimatization before the study start. Standard conditions of temperature, regular 12 h light/dark cycle and free access to standard rat food were considered. A random equal classification of rats was done, as following:

1. Normal control group: rats received 0.2 ml phosphate buffer saline (PBS) (10 mM, pH 7.4) by i.p. injection twice weekly, for 16 weeks.
2. AntiGPC-3 treated Control group: rats received 1.2 μg/kg unconjugated polyclonal IgG fraction of antiserum, lyophilized powder of antiglypican-3 (antiGPC-3), (Sigma-Aldrich Chemicals Co., St Louise, MO, USA) in 0.2 ml of PBS once weekly by i.p. injection for 16 weeks.
3. HCC group: rats received 200 mg/kg of thiocetamide (Sigma-Aldrich Chemicals Co., St Louise, MO, USA) to induce HCC in 0.2 ml PBS by i.p. injection twice weekly for 14 weeks (Dasgupta et al., 1981). In week 16th, a blood sample was

Fig. 1. AntiGPC-3 antibody blocked GPC3 levels in vivo. A) Statistical analysis showed significant increase in GPC3 in HCC patients in comparison to cirrhotic patients or controls, respectively. B, C) Statistical analysis and representative images of rat livers stained with antiGPC-3 showing significant increase in hepatic GPC3 in HCC rats as compared to the control group. Treatment with antiGPC-3 significantly reduced GPC3 in HCC rats and did not affect the control group. *Significant difference as compared with all groups at P < 0.05. **Significant difference as compared with the control groups at P < 0.05. AntiGPC-3: antiglypican-3, HCC: hepatocellular carcinoma, C: control.
withdrawn; and serum AFP level was determined using ELISA kit (DiaMetra S.r.i, Italy) to confirm HCC induction.

(4) AntiGPC-3 treated HCC group: rats received 1.2 mg/kg antiGPC-3 in 0.2 ml PBS by i.p. injection once weekly for the first two weeks, continued simultaneously with 200 mg/kg of thioacetamide in 0.2 ml PBS i.p. injection twice weekly for the rest 16 weeks.

2.3. Collection of animal samples

At the end of the study period, rats were denied food for 8 h and only allowed access to water. Rats were killed by decapitation. Blood samples were collected and centrifuged at 4000 rpm for 5 min and the sera were separated and stored at −80 °C. Rats' livers were removed, weighed and divided to three sections; first section was fixed in 10% buffered formalin for morphological analysis. The second section was homogenized in PBS. Last section was immediately immersed in liquid nitrogen and stored at −80 °C for quantitative RT-PCR analysis.

2.4. Morphological study of rat liver tissue

The liver sections were fixed in 10% buffered formalin and embedded in paraffin. 5 μm-thick sections were cut and stained with Mayer's hematoxylin and eosin (H&E) for examination of cell structure by a light microscope. Liver specimen was anonymously coded and examined in a blinded manner. The morphological changes were photographed using a digital camera-aided computer system (Nikon digital camera, Japan).

2.5. Immunohistochemistry

Immunohistochemical (IHC) analyses were performed on 5-μm-thick paraffin sections cut from a paraffin block of liver. Sections were incubated over night with antiGPC-3 (Sigma-Aldrich Chemicals Co., St. Louis, MO, USA) and anti-IGF-II (Abcam, Cambridge, MA, USA) at 4 °C. Then, sections were incubated with horseradish peroxidase conjugate antibody. The chromogen used was 2% 3, 3′-diaminobenzidine (DAB) in 50 mM Tris-buffer (pH 7.6). Slides were counterstained with hematoxylin and photographed using a digital camera-aided computer system (Nikon digital camera, Japan).

2.6. Evaluation of the anti-tumor profile of antiGPC-3

Anti-tumor activity of antiGPC-3 was evaluated through:

Animal survival was determined at the end of the experiment by calculating the percent of surviving rats in each group to the total starting number of rats in the group. Serum AFP was determined using ELISA kit (DiaMetra S.r.i, Italy) according to the manufacturer protocol.
2.7. Liver function assay

Serum alanine transferase (ALT) activity (Spectrum Diagnostics, Germany), albumin and bilirubin concentrations (Diamond Diagnostics Co., USA.) were measured using standard colorimetric methodologies by commercially available kits.

2.8. Assessment of oxidative stress

Liver homogenates were used for measuring Superoxide anion (Baehner et al., 1976), malondialdehyde (MDA) (Satoh, 1978), reduced Glutathione (GSH) (Eyer and Podhradsky, 1986) and superoxide dismutase (SOD) levels (Marklund and Marklund, 1974) (Sigma-Aldrich Chemicals Co., St.Louise, MO, USA).

2.9. Enzyme-linked immunosorbent assay (ELISA)

GPC3, HSPG and SULF-2 (Uscn Life Science Inc., USA) and IGF-II (BioSource Europe Company, Belgium) were measured using commercially available ELISA kit according to the manufacturer protocols.

2.10. Quantitative, real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from rat liver using TRIzol® Reagent (Life Technologies, USA). The yield and quality of total RNA were determined spectrophotometrically at 260 and 260/280 nm ratio, respectively. The amount of RNA was quantified by using a Maxima® SYBR Green/Fluorescein qPCR Master Mix (Fermentas, USA). 1 μg of total RNA was reverse-transcribed into single-stranded complementary DNA by using Quantitect® Reverse Transcription Kit (Qiagen, USA) using a random primer hexamer in a two-step RT-PCR reaction in which any genomic DNA (gDNA) contamination was eliminated using gDNA Wipeout buffer. Caspase-3 and IGF-II mRNA in different rat liver tissues was determined using Maxima® SYBR Green/Fluorescin qPCR Master Mix by Rotor-Gene Q (Qiagen, USA). Meanwhile, rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene and an internal reference control. Gene specific PCR primers (Table 2) were designed using Primer Express 3.0 (Applied Biosystems, USA) according to the nucleotide sequence obtained from the Gene Bank. Thermal cycling conditions included initial activation step at 95 °C for 10 min followed by 40–50 cycles at 94 °C for 15 s, 55 °C or 58 °C for 30 s and 72 °C for 1 min. Data acquisition was performed during the extension step. Melting curve analysis of the PCR product(s) was performed to verify their specificity and identity. Rotor-Gene Q collected data automatically and analyzed the value of threshold Cycle (Ct). Rat caspase-3, IGF-II and GAPDH mRNA relative expression was determined by using a 2–ΔΔCt method. PCR products were confirmed by running on 1.2% agarose gel electrophoresis.

2.11. Statistical analysis

For descriptive statistics, frequency and percentage were calculated for qualitative variables, the mean ± S.E.M. For comparison of means between groups, one-way analysis of variance (ANOVA) was used.
Once the differences exist among the means, post-hoc Bonferroni correction test was calculated. Kaplan–Meier method was used for calculation of rats’ survival. Statistical computations were done using Excel 2007 and statistical significance was predefined as $P \leq 0.05$.

### 3. Results

#### 3.1. AntiGPC-3 antibody blocked GPC3 levels in vivo

In human subjects, serum level of GPC3 was significantly high in HCC patients when compared to cirrhotic and control groups ($P < 0.05$) (Fig. 1A). In parallel, HCC rats showed significant 1.8-fold increase in GPC3 level when compared to control rats as well as increased GPC3 in hepatocytes in rat sections stained with antiGPC-3. Treatment of HCC rats with antiGPC-3 blocked elevated GPC3 levels in HCC rats without affecting the control rats (Fig. 1B and C).

#### 3.2. AntiGPC-3 antibody partially reduced HCC-induced elevation in oxidative stress markers and restored some of the antioxidant activity

Rats with HCC showed significant increases in hepatic levels of MDA and superoxide anion (4.2- and 1.9-fold, respectively) and reduced GSH and SOD levels when compared to control rats. AntiGPC-3 partially but significantly blocked the elevated hepatic MDA and superoxide anion concentrations in HCC group and did not affect the control group (Fig. 2A and B) as well as significantly elevated hepatic levels of GSH and SOD (Fig. 3A and B).

#### 3.3. AntiGPC-3 antibody reduced HCC-induced levels of SULF-2 and restores HSPG

Serum HCC groups showed significant increase in SULF-2 as well as significant reduction in HSPGs as compared to cirrhotic patients or control group (Fig. 4A and C). In parallel, treatment of

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**Fig. 5.** AntiGPC-3 antibody blocked HCC-induced expression of IGF-II.

A. Statistical analysis showed significant increase in IGF-II protein level measured by ELISA in HCC patients compared to cirrhotic and control groups. B) Statistical analysis of gene expression of IGF-II in hepatic homogenate showed significant reduction of IGF-II in rats treated with antiGPC-3 compared to HCC-untreated group. C) Representative images of rat livers stained with anti-IGF-II showing significant elevation in HCC rats that was reduced by treatment with antiGPC3. *Significant difference as compared with the rest of the groups at $P < 0.05$. AntiGPC-3: antiglypican-3; HCC: hepatocellular carcinoma; IGF-II: insulin-like growth factor-II.
HCC rats with antiGPC-3 showed significant reduction in SULF-2 and elevation in HSPGs as compared with HCC rats and did not affect the control rats (Fig. 4B and D).

3.4. AntiGPC-3 antibody blocked HCC-induced expression of IGF-II

A significant increase in serum IGF-II level was detected in HCC patients as compared to control group or cirrhotic patients ($P < 0.05$). However, cirrhotic group showed no significant difference as compared to control group (Fig. 5A). However, HCC rats showed significant increase in the gene expression of IGF-II in hepatic homogenate. Moreover, liver sections stained with IGF-II showed increased hepatocytes levels of IGF-II in HCC rats as compared with control rats, which was blocked by treatment with antiGPC-3. AntiGPC-3 treatment did not affect the control rats (Fig. 6).

3.5. AntiGPC-3 antibody blocked HCC-induced elevation in gene expression of caspase-3 in rats

HCC rats showed significant increase in caspase-3 gene expression as compared with the control group. Treatment of HCC rats with antiGPC-3 significantly reduced caspase-3 gene expression in HCC rats and did not affect the control rats (Fig. 6).

3.6. Anti-tumor activity of antiGPC-3 antibody

Animal survival was calculated after 16 weeks following antiGPC3 treatments at the end of the experiment. The percent of rats’ survival was 40% in HCC group (Fig. 7A). However, treatment with antiGPC3 elevated rats’ survival up to 90%. To confirm the results, antiGPC3 significantly decreased the elevated AFP in HCC-treated group and did not affect the control group (Fig. 7B).

3.7. Hepatoprotective effects of antiGPC-3 antibody

GPC3 plays an inhibitory role during rat liver regeneration and hepatocyte proliferation (Liu et al., 2010). A routine evaluation of liver function markers was tested to show if antiGPC3 possesses hepatoprotective effect. HCC group showed 2.24-fold increase in serum ALT activity and 1.8-fold increase in bilirubin concentration as well as 46% reduction in albumin concentration when compared to normal control group. All these effects were improved by treatment with antiGPC-3. In addition, liver sections stained with H&E showed marked cellular infiltrate, fibrosis and breakdown of hepatic tissues. However, sections from HCC rats treated with antiGPC3 showed nearly normal appearance of hepatic lobule (Fig. 8B).

4. Discussion

HCC has a rising rate of incidence worldwide, and especially in Egypt. An urgent need for new treatment options emerged because of the late diagnosis. Although, Sorafenib is the only medication that shows overall survival advantage compared to placebo in patients with advanced HCC, its moderate benefits and a challenging toxicity make surgery the standard treatment (Llovet et al., 2008; Scanga and Kowdley, 2009).

Over the past few years, several mouse monoclonal antibodies against GPC3 have been produced (Ho, 2011) such as humanized GC33 (hGC33), which is under evaluation in a phase I clinical trial (http://clinicaltrials.gov/ct2/show/NCT00746317). hGC33, a monoclonal antibody against the COOH-terminal part of GPC3, was proven before to be efficacious against the HepG2 xenograft and a potential antitumor agent for human liver cancer (Nakano et al., 2010). One proposed mechanism of antitumor activity of GC33 was antibody-dependent cellular cytotoxicity. However, the involvement of other mechanisms such as modulation of the functions of GPC3 in anti-tumor activity remains to be elucidated (Ishiguro et al., 2008). Another possible mechanism was studied when HN3 was developed. HN3 is a human heavy-chain variable domain...
antibody. HN3 showed ability to inhibit proliferation of GPC3-positive cells and exhibited significant inhibition of HCC xenograft tumor growth in nude mice. The underlying mechanism of HN3 action was hypothesized to involve cell-cycle arrest at G1 phase through Yes-associated protein signaling (Sun et al., 2011).

In this study, we hypothesized that another mechanism of antiGPC-3 was attributed to block of HSPG/SULF-2/IGF-II axis (Fig. 9). We found significant increase in serum GPC3 in HCC patient compared with the control group, which was consistent with the speculation of serum GPC3 to be a selective promising potential marker for HCC (Boudin et al., 2013; Chen et al., 2014; Miao et al., 2014; Witjes et al., 2013; Yao et al., 2013). Therefore, we evaluated the usefulness of targeting GPC3 using AntiGPC-3 antibody in rats. Treatment with antiGPC-3 was found to have an anti-tumor activity as it improved the percent survival of the rats up to 90% compared with only 40% in HCC group. In addition, antiGPC3 significantly reduced serum AFP in HCC group. Many pathways involving GPC3 were identified to promote the growth of HCC by stimulating canonical Wnt signaling (Boudin et al., 2013; Capurro et al., 2005a), fibroblast growth factor and transforming growth factor-β signaling pathways during development (Baeg and Perrimon, 2000). However, antiGPC-3 was found to almost block the hepatic level of GPC3 in HCC rats; which is consistent with previous finding regarding reduced GPC3 levels, where GPC3 knockdown was able to inhibit cell proliferation in the HCC cell lines Huh-7 and HepG2 (Sun et al., 2011). Of note, we found

![Graph showing statistical analysis](image)

**Fig. 8.** Hepatoprotective effects of antiGPC-3 antibody. A) Statistical analysis showed significant decrease in liver function markers; ALT, albumin and bilirubin in HCC-treated group with antiGPC-3 when compared to HCC-untreated rats. B) Histopathological examination of both control and HCC tissues with/without antiGPC-3 treatment stained by H&E. Sections from the control and treated control group showing normal hepatocytes with some appear binucleated HCC cessed disrupted hepatocytes and many appear with vacuolated cytoplasm. Cellular infiltrate is present between hepatocytes. Sections from rats treated with anti-GPC3 showing nearly normal appearance of hepatic lobule. Some hepatocytes appear with dark nuclei. *Significant difference as compared with the rest of the groups at P < 0.05. #Significant difference as compared with the control groups at P < 0.05. AntiGPC-3: antiglypican-3; HCC: hepatocellular carcinoma; ALT: alanine transaminase, C: control.
previously that antiGPC3 significantly reduced viability and increased cytotoxic activity of HepG2, human HCC cell-line, in a dose dependent manner (Randa et al., 2013).

Following the GPC3 pathway, GPC3 may confer oncogenicity through interacting with IGF-II and its receptor and the activation of the IGF-signaling pathway involving ERK pathway (Cheng et al., 2008). We found significant increase in serum IGF-II in HCC patients. It was previously proven that the increase in IGF-II expression has been observed in liver cancers including HCC (Cheng et al., 2008). In parallel, HCC rats showed highly elevated gene expression of IGF-II in HCC. IGF-II is a polypeptide hormone secreted by many organs of the fetus; however, its expression is strictly down regulated shortly after birth (Nussbaum et al., 2008). IGF-II is a growth factor that plays an important role during HCC and its expression has been confirmed in the liver tissues of HCC (Breuhahn et al., 2006; Nussbaum et al., 2008). The levels of IGF-II expression suggest that it may be secreted by hepatoma cells themselves and stimulate their proliferation via an autocrine mechanism. IGFs are potent autocrine and paracrine mitogens for liver cancer cell proliferation. Human embryonic liver cell lines also express IGF-II, suggesting that hepatoma cells may regain some embryonic characteristics like AFP secretion (Himoto et al., 2005). In addition, serum IGF-II protein was significantly higher in the HCC group than in control groups. When hepatocytes are transformed into malignant cells, they may secrete IGF-II and promote malignant cell proliferation by an autocrine mechanism (Wang et al., 2003). The expression profile of IGF-II in liver sections stained with anti-IGF-II was associated with the morphological changes of hepatocytes. Positive staining of hepatic IGF-II showed brown particles, located in cytoplasm and cellular nuclei but none in cell membrane. Positive cells were mostly located in the margin of portal area or near the central vein. The expression level of IGF-II went up with the histological changes. It was significantly higher in the HCC or the surrounding group than in the noncancerous group.

SULT-2 was previously shown to be highly expressed in HCC as it increased cell surface GPC3 expression (Lai et al., 2010). Of note, we found significant increase in serum SULT-2 activity in HCC patients when compared to cirrhotic patients or control group. In addition, antiGPC-3 was found to significantly reduce SULT-2 in HCC rats. Moreover, antiGPC-3 significantly elevated HSPGs in HCC group, proving that SULT-2 level was decreased by antiGPC-3 in rats. OKN-007, a novel phenyl-sulfonlyl compound that inhibits the enzymatic activity of SULT-2, repressed tumor growth significantly suggesting that SULT-2 inhibitor derivatives may be promising agents for the treatment of HCC (Zheng et al., 2013). However, antiGPC-3 has no direct action on SULT-2 (Randa et al., 2013). The heptoprotective effects of antiGPC-3 against HCC-development in treated rats, which can decrease HCC-induced SULT-2 overexpression, can explain this. In addition, IGF-II was reported to increase the expression of steroid sulfatase mRNA and protein in concentration- and time-dependent manners (Sung et al., 2013). Therefore, the reduced IGF-II expression by antiGPC3 treatment can reduce the activity of SULT-2. However, further studies should be carried out.

AntiGPC-3 did not only demonstrate antitumor activity but hepatoprotective activity as well. AntiGPC-3 improves liver function markers; ALT, total bilirubin and albumin. In addition, antiGPC3 prevented the hepatocytes break down and the massive fibrosis found in the HCC rats. In addition, serum AFP was lower than the cutoff value of HCC. This may explain the next finding, where antiGPC-3 was surprisingly able to reduce gene expression of caspase-3. AntiGPC-3 was found to reverse the apoptotic cascade related to the chronic liver damaged proven by the decreased in the expression of caspase-3, leading to protection of the hepatocytes. To nullify this reduction in caspase-3 expression by the indirect cause of hepatoprotective effect of antiGPC-3, we measured caspase-3 activity in well developed HCC cell line, hepG2, where antiGPC-3 increased caspase-3 activity consistent with others (Liu et al., 2012). Of note, oxidative imbalance was well established in HCC patients consistent with others (Eissa et al., 2013).

5. Conclusions

The main findings of the current study are that antiGPC3 possesses chemopreventive and hepatoprotective effects. The hypothesized mechanism of action is summarized in Fig. 9. Targeting GPC3 is useful in combating HCC through several mechanisms; as inhibition of oxidative stress, IGF-II, and caspase-3. We found for the first time that anti-GPC3 reduced hepatic gene expression of both IGF-II and caspase-3 and the activity of sulfatase-2. However, the chemopreventive effects of anti-GPC3 in the current study were demonstrated using anti-GPC3 as a prophylactic agent. Therefore, chemopreventive and hepatoprotective effects of anti-GPC3 in treating rats with HCC warrant investigation.

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References


