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Cytotoxic effects of antiglypican-3 against HepG2 cell line

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ABSTRACT

For decades, various systemic therapies have been explored for the treatment of advanced hepatocellular carcinoma (HCC), the fifth most common cancer and the third leading cause of cancer-related deaths worldwide. Nevertheless, no satisfactory results have been obtained so far. Glypican-3 (GPC-3) is a cell-surface heparan sulfate proteoglycans (HSPGs) that emerged as a promising diagnostic marker as well as target for therapy. Therefore; we investigated antitumor activity of antiglypican-3 (antiGPC3), a specific antibody aginst GPC-3, against HepG2, human HCC, cell line. HepG2 cells were treated with AntiGPC3 at (5, 10 and 20 µg/ml). HepG2 cell proliferation was measured by MTT and lactate dehydrogenase (LDH) assays. GPC-3, HSPG and sulfatase-2 (SULF2) levels were measured by ELISA. Moreover, apoptosis was assessed by measuring Caspase-3 activity. We found that, antiGPC3 reduced HepG2 cells survival and showed cell cytotoxicity in a dose-dependent manner. In addition, antiGPC3 was able to increase the apoptosis measured by capase-3 activity in hepG2 cells. Finally, antiGPC3 restored HSPG level without affecting SULF2 in HepG2 cells. We can conclude that, antiGPC3 possesses cytotoxic effects, which can be partially explained by restoration of HSPGs and increase of caspase-3 apoptotic pathway. GPC-3 represents a promising target of HCC therapy.

INTRODUCTION

Hepatocellular carcinoma (HCC), a primary liver cancer, is one of the most frequent tumors representing the fifth commonest malignancy worldwide and the third cause of mortality from cancer. Unfortunately, the overall response rate of liver cancer treatment is unsatisfactory mainly due to late diagnosis and poor treatment efficacy, especially resistance to chemotherapeutic drugs and metastasis to other organs (Metwaly *et al.,* 2012). Hepatectomy offers the best outcomes for patients with HCC. 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 (Monga *et al.,* 2013). Glypican-3 (GPC-3) is a member of glypican family of glycosyl-phosphatidylinositol-anchored cell-surface heparan sulfate proteoglycans (HSPG) (Chen *et al.,* 2013). GPC-3 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 (Zhu *et al.,* 2001; Nakatsura *et al.,* 2003; Hippo *et al.,* 2004; Capurro *et al.,* 2005; Nishimura *et al.,* 2008).

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Sulfatase-2 (SULF2) is an enzyme with 6-O desulfatase activity on Sulfated heparan sulfate glycosaminoglycan (HSGAG) chains of GPC-3 and other HSPG. SULF2 is upregulated in primary HCC and associated with a worse prognosis (Lai *et al.,* 2010). The interaction between SULF2 and GPC-3 revealed that forced expression of SULF2 enhanced GPC-3 expression both in vitro and in vivo (Chen *et al.,* 2013). Therefore, the study investigated the possible antitumor activity of targeting GPC-3 by antibodies, antiglypican-3 (antiGPC3), in HHC cell line, hepG2, in three different doses (5, 10 and 20 µg/ml), proving GPC-3 to be a possible target for immunetherapy for HCC.

EXPERIMENTAL

Cell lines

Human hepatocellular carcinoma (hepG2) cell line was purschased (VacSERA, Egypt). hepG2 cell line was ensured to be mycoplasma-free through microbiological culture. HepG2 cells were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and 1% streptomycin and penicillin, and incubated for 24 hr at 37°C in a 5% CO_2 incubator to allow the cells to grow. 1×10^4 cells were plated in each well of 96-well plates, and were placed in the humidified 5% CO_2 incubator at 37°C to allow them to grow for 24-h period.

Cells were exposed to different concentrations of antiGPC3 (5, 10 and 20 µg/ml) and placed in the humidified 5% CO_2 incubator for 48 h. Cells incubated in culture medium alone served as a control for cell viability (untreated wells). Each experiment was repeated at least three times. After antiGPC3 treatment, cells were aspirated by 500 µL of phosphate buffer saline (PBS) (10 mM, pH 7.4) and centrifuged for 5 min at 4000 r.p.m to remove cell debris and obtain a clear supernatant.

MTT assay

Cell viability was determined using the MTT assay as described previously (Al-Gayyar *et al.*, 2011).

Evaluation of cytotoxicity with LDH

Equal Supernatant was plated in a 96-well plate. LDH solution was added to each well including controls and cell-free wells. The plate was allowed to develop for 20 min in the dark at room temperature. Cytotoxicity with LDH was determined by subtracting the normalized absorbance at 680 nm of the cell-free wells from the normalized absorbance of wells with cells. Relative cytotoxicity was determined by normalizing against the positive cytotoxicity control, 1% Triton-X.

Assessment of oxidative stress

The oxidative stress was estimated in hepG2 cell lines through measurement of hydrogen peroxide level (Davies, 1999) and superoxide dismutase (SOD) activity (Marklund and Marklund, 1974).

Enzyme-linked immunosorbent assay (ELISA)

GPC-3, HSPG and SULF2 levels were measured using commercially available ELISA kit (Uscn Life Science Inc., USA) following the manufacturer procedure.

Estimation of caspase-3 activity

Caspase-3 enzyme activity assay was measured colorimetrically using commercially available kit (GenScript, Piscataway, NJ, USA) following the manufacturer procedure.

Statistical analysis

The mean values ± standard error was used for quantitative variables. For comparison between two groups student t-test was used. Statistical computations were done on a personal computer using the computer software SPSS version 13 (Chicago, IL, USA). Statistical significance was predefined as $P \leq 0.05$.

RESULTS AND DISCUSSION

Due to the limited treatment options for advanced HCC, HCC has a poor prognosis. Although Sorafenib, an oral multi-kinase inhibitor, has emerged to be an efficacy therapy as well as the standard drug for first-line systemic treatment (Llovet *et al.*, 2008), No second-line treatment has been established for patients who do not respond to Sorafenib treatment. Therefore, new treatment modalities are urgently required to prolong the survival of patients with advanced HCC. 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). Recently, antigen-specific cancer immunotherapies against HCC, including peptide vaccines, dendritic cell vaccines and adoptive cell transfer therapies have attracted much interest (Olioso *et al.*, 2009; Greten *et al.*, 2010).

Glypican-3 (GPC-3) is an ideal target for anticancer immunotherapy against HCC because it is specifically overexpressed in HCC and correlates with poor prognosis (Filmus, 2002). The biological functions of GPC-3 and its role in liver tumorigenesis remain elusive. In this study, it was confirmed the ability of antiGPC3 to reduce the levels of GPC-3 in HepG2 cell lines (Fig. 1). We investigated 3 different doses of antiGPC3 (5, 10 and 20 µg/ml). The use of antiGPC3 resulted in a significant dose-dependent decrease in the cell survival as indicated by MTT assay as well as significant dose-dependent elevation of cytotoxicity as indicated by LDH level in relation to Triton-X (Fig. 2). It was reported previously that, the use of GPC-3 derived peptide vaccination against advanced HCC demonstrated the induction of peptide-specific cytotoxic T lymphocytes (CTLs) and significantly correlated with patient survival (Sawada *et al.*, 2012). In transgenic mice, overexpression of GPC-3 suppresses hepatocyte proliferation and liver regeneration (Liu *et al.*, 2010; Bret *et al.*, 2011).

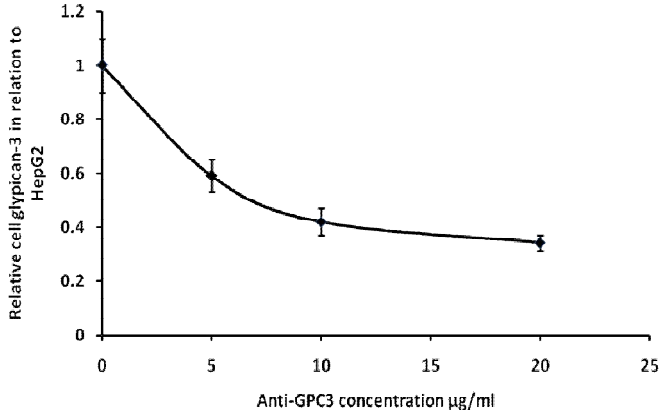
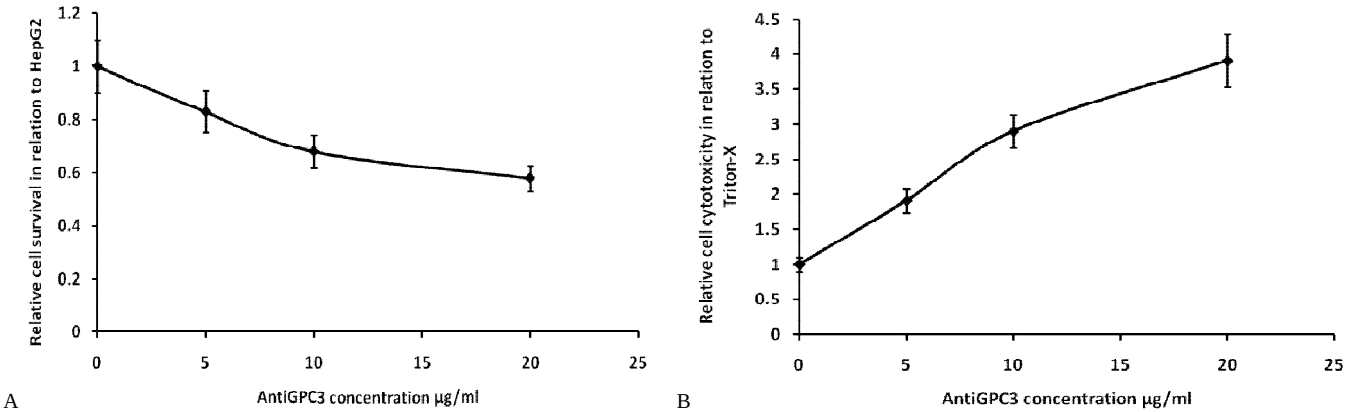


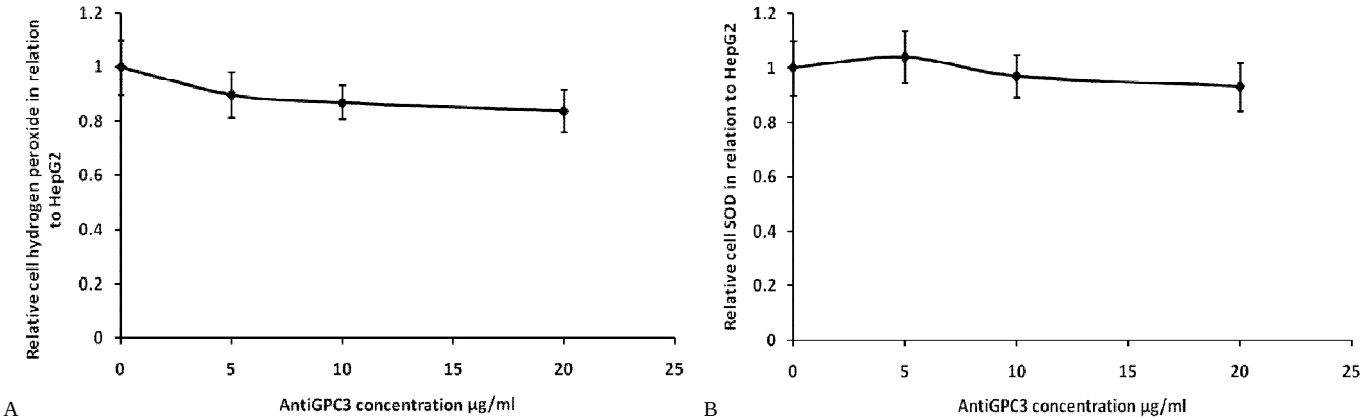
Fig 1: Effect of different doses of antiGPC3 (5, 10, 20 µg/ml) on glypican-3 (GPC-3) level in HepG2 cells.

The antitumor activity of antiGPC3 was hypothesized to be due to an improvement on the oxidative stress imbalance. However, antiGPC3 showed no effect on both hydrogen peroxide and superoxide dismutase revealing that its mechanism of action is independent of the oxidative stress (Fig. 3). Next we examined the effect of antiGPC3 on heparan sulphate proteoglycan (HSPG) and sulfatase-2 (SULF2) levels. We found that, treatment of HepG2 cells with antiGPC3 showed dose-dependent elevation of HSPG with no significant effects on SULF2 levels (Fig. 4). It has been reported previously that, the expression patterns of proteoglycans including HSPGs are markedly changed under pathological conditions (Krull and Gressner, 1992).



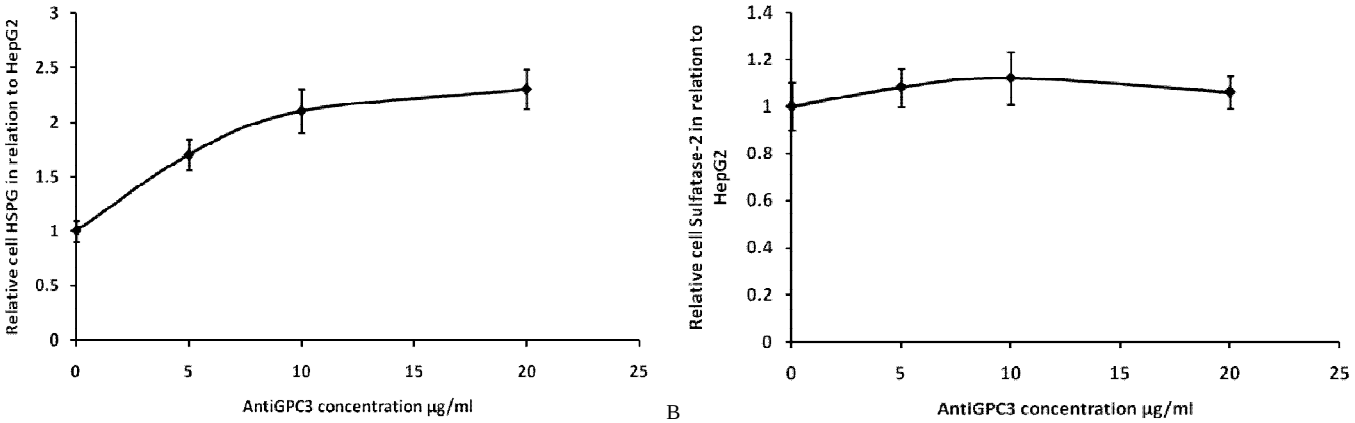
A B

Fig. 2: Effect of different doses of antiGPC3 (5, 10, 20 µg/ml) on cell survival (A) and cell cytotoxicity (B) in HepG2 cells.



A B

Fig. 3: Effect of different doses of antiGPC3 (5, 10, 20 µg/ml) on hydrogen peroxide level (A) and superoxide dismutase (SOD) activity (B) in HepG2 cells.



A B

Fig 4: Effect of different doses of antiGPC3 (5, 10, 20 µg/ml) on heparan sulfate proteoglycans (HSPG) level (A) and sulfatase-2 (SULF2) activity (B) in HepG2 cells.

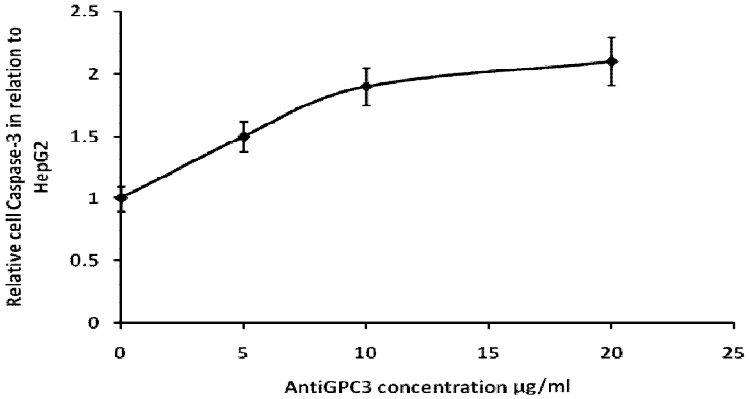


Fig. 5: Effect of different doses of antiGPC3 (5, 10, 20 µg/ml) on caspase-3 activity in HepG2 cells.

HSPGs are negatively-charged proteins located at a high cell density on various cell types or released into the ECM (Chen *et al.*, 2013; Fu *et al.*, 2013). As HSPGs bind a large diversity of molecules: growth factors, cytokines, chemokines, morphogens, matrix ligands and cell surface molecules, they are involved in cell signaling as co-receptors (Bret *et al.*, 2011). These are in agreement of previous studies reported the expression of several HSPGs including glypican-3 (Capurro *et al.*, 2003), syndecan-4 and perlecan (Charni *et al.*, 2009) are increased in HCC tissues. They are thought to promote tumor growth, invasion, and angiogenesis (Blackhall *et al.*, 2001; Fuster and Esko, 2005; Fears and Woods, 2006).

Finally, to elucidate the molecular mechanisms underlying antiGPC3 induced antitumor activity, caspase-3, an apoptotic marker, was assessed. As observed in figure 5, treatment of HepG2 cells with antiGPC3 resulted in a significant elevation of caspase-3 activity by all three doses (5, 10 and 20 µg/ml) compred to contol. The dose 20 µg/ml of antiGPC-3 caused the highest elevation. It was found that silencing glypican-3 expression induces apoptosis in human hepatocellular carcinoma cells (Liu *et al.*, 2012). Whereas, it was suggested that GPC-3 regulates cell proliferation by enhancing the resistance to apoptosis through the dysfunction of the Bax/Bcl-2/cytochrome c/caspase-3 signaling pathway and therefore plays a critical role in the tumorigenesis of HCC (Liu *et al.*, 2012).

CONCLUSIONS

The main findings of the current study are that antiGPC3, antibody targeting GPC-3, possesses cytotoxic effects, which can be partially explained by restoration of HSPGs and activation of caspase-3 apoptotic pathway. However, antiGPC3 has no effects on oxidative stress status or SULF2 level. Therefore, GPC-3 represents a promising target for HCC therapy.

AUTHORS' STATEMENT

The authors declare no conflict of interest.

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