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Research Article

Prognosis of Hepatocellular Carcinoma with the Detection of Glypican 3 and its Inhibition by Triptolide using Docking Studies with Autodock Vina

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ABSTRACT

Intake of fluoridated water can be one of the most common neglected factors affecting the livers of individuals. Hepatocellular Carcinoma (HCC) is the most common malignant tumor responsible for a large number of deaths globally. Surveillance requires the early detection of HCC among patients at the first stage of cancer. The study aimed at the prognosis of HCC for which the Glypican-3 (GPC3) biomarker was used. Fluoride content in drinking water and serum of populations from district Narowal (sample) and DHA Lahore (control) was measured by the ion-selective electrode. The levels of expression of *GPC3* in both samples and controls were determined by ELISA. The binding affinity of triptolide to glypican 3 using AutoDock Vina was determined by hydrogen bonds, binding energy, and clusters of interacting amino acid residues using discovery studio. Results analyzed by student's t-test showed a significant increase ($p=0.0001$) in *GPC3* with rising fluoride content in sample water and serum samples. Ligand binding modes with glypican3 showed the highest binding affinity (Δ -7.1 kcal/mol) with the lowest RMSD (0.000) by Autodock Vina. The study proves that the long-term consumption of fluoridated water is one of the leading causes of HCC that can be diagnosed at early stages by the detection of glypican 3 levels. Further, binding of triptolide to glypican3 in nonspecific manner demands structurally modified pharmacological targets for the prevention of HCC.

Keywords: Fluoride toxicity; Hepatocellular carcinoma; Glypican 3; Docking

INTRODUCTION

In humans, fluorine supports dental health, but in high concentrations, it quickly becomes toxic-leading to bioaccumulation in tissues. Dental fluorosis, skeletal fluorosis, arthritis, osteoporosis, and muscular damage are well-defined sequelae to toxic levels of Florine. Accumulations are also seen in the myocardium, arteries, nephron, liver, endocrine glands, and nervous system [1,2]. Significant effects are seen on the human immune, reproductive, gastrointestinal, and urinary systems of consuming greater than 1.0 mg/L of fluorine in water [3]. Fluorine also augments the toxicity of other heavy metals by binding with them and altering their excretion rates [4].

High concentrations of fluoride lead to histopathologic alterations in the liver [5]. Liver cells of rats exposed to high concentrations of fluoride show changes in proteins related to the endoplasmic reticulum, mitochondria, apoptosis, and cellular respiration [6]. In addition to these issues, another study reported changes in liver microstructure and the expressions of immune-related genes in Zebrafish [7]. Microstructural alterations in the liver increase with the concentration of fluoride as well as the duration of exposure. High fluoride is also linked to an increase in the apoptotic index and an upregulation of the Bax/Bcl2 apoptotic factor [8].

High fluoride has been reported for its ability to increase the expression of *GRP78* in rat liver, which denotes endoplasmic

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reticulum stress and protein misfolding. In a novel study on the proteomic analysis of liver cells chronically exposed to fluoride, forty-nine proteins related to metabolism were found significantly altered. In addition, proteins related to liver injury were found to increase, and those having antioxidant roles were depleted [9]. This all points to oxidative stress on the liver, and certain damage at a cellular level. Evidence of epigenetic changes is also reported, with disruption of DNA methylation seen with higher Fluoride exposure [10].

Literature as early as 1991 reported mild carcinogenic activity of fluoride in male rats [11]. It was further supported by a study in 1993 showing the promotive effect of fluoride on precancerous lesions of hepatocellular carcinoma in rats [12]. A study conducted to test the carcinogenicity of substances based on DNA-damage-induced gene expression in human HepG2 cells found sodium fluoride to be among the carcinogens [13]. According to one study in Colombo, rats that ingested water with fluoride levels up to 1.3 mg/L had a higher incidence of Hepatocellular Carcinoma [14]. However, little research is done to compare fluoride concentrations in drinking water with the symptomatic disease of hepatocellular carcinoma in humans. This is the first aim of our study-to investigate fluoride-induced hepatocellular carcinoma, using serum levels of Glypican-3 in the rural areas of district Narowal, Punjab, chronically exposed to high levels of fluoride in drinking water.

Hepatocellular carcinoma is 5th most commonly diagnosed carcinoma globally [15]. Glypican-3 is being used as a reliable biomarker for the early detection of hepatocellular carcinoma [16]. Pakistan reports high concentrations of fluoride in drinking water. A 2021 study conducted in Thar calculated the fluoride content of well water to be 6-8 mg/L [17]. In many rural parts of Pakistan, well water is consumed for drinking purposes. This challenges Pakistan to assess and counter any damage caused to these exposed populations.

GPC3 is a member of the glypican family and its expression is normally detected in the placenta, numerous embryonic tissue the mammary gland, adult ovary, lung, kidney, and mesothelium [18,19] . In healthy adult liver, no *GPC3* expression is detected, however, its overexpression has been detected in HCC. Further, *GPC3* protein and gene expression levels in the serum and tumor tissue are higher in HCC than in healthy or non-malignant livers [20]. Glypican 3 promotes growth of HCC cells *via* Wnt signaling pathway [21]. It interacts with Wnt ligands and stimulates cell proliferation [22]. Another study reported the inhibition of HCC cell growth and proliferation by transforming growth factor β receptor (TGF-β2) in siRNA-GPC3-mediated growth suppression [23]. In an *in-vitro* experiment, small interference RNA with a glypican 3 small hairpin RNA was used to identify the effects of *GPC3* in the malignant behavior of HCC on highly metastatic HCC cell lines (MHCC97-H). The authors reported that there is a significant role of glypican 3 in the malignant behavior of HCC [24].

Triptolide is an extract of Chinese herb, Tripterygiumwilfordii Hook F (TWHF) and is known for its clinical effects among four hundred components derived from TWHF. It has been reported as anti-immunosuppressive, anticancer, anti-inflammatory, anti-fertility, anticystogenesis, and chemoprotecting natural bioactive compound [25,26]. Commercial preparations are widely used to treat autoimmune and inflammatory diseases [27].

To our knowledge, no study has been carried out to investigate the binding affinity and inhibitory effects of triptolide to glypican 3 using computational tools. Since glypican 3 is a novel target for the clinical manifestation of HCC, our present study elucidated the mechanism of binding affinity of triptolide to glypican 3 using computational tools, AutoDock Vina.

MATERIALS AND METHODS

Place of the study

This study was conducted in the department of Biochemistry, Minhaj University, Lahore from March 2020 to January 2021. Ethical approval was obtained from the institutional ethical committee at Minhaj University, Lahore.

Subjects

Male and female aged between 18 to 65 were included in the present study. Patients having recurrent complains of symptoms like vomiting, decreased appetite, excessive weight reduction, and yellow skin, abdominal pain consuming well water of Narowal for five years or more were included in the study after taking written informed consent. People aged 66 and above were not included in the study. Patients already diagnosed with HCC, HCV, HCB, under prolonged use of medications or exposed to alcohol or nicotine were also excluded. A total of 100 males and females were included in the study. Subjects were categorized into control subjects (n=30) from DHA and tests from Narowal (n=70). Each individual was subjected to written informed consent.

Collection of water samples

Water samples were collected from district Narowal for test and from DHA, Lahore for control and stored at 5°C to check fluoride levels.

Collection of blood samples

Venous blood sampling was performed obtaining 7 ml of blood by venipuncture. 4 ml was collected in a vial containing no additive and 3 ml of blood was collected in an EDTA vial to obtain plasma rather than serum for the estimation of fluoride contents from blood. The serum was extracted from blood samples by centrifuging blood at 3000 rpm for 15 minutes and was preserved at -20°C for further analysis

Estimation of fluoride contents in water and blood samples

Fluoride concentrations in water and blood samples were determined using the ion-specific electrode (fluoride electrode-Orion, 9409). Its basic principle refers to the method established by Kissa [28]. The limit of detection of the electrode was 0.019 mg/L.

Estimation of glypican 3

Glypican 3 was estimated using an ELISA kit (creative diagnostic, USA: DEIB BJ188) This assay employs the quantitative sandwich enzyme immunoassay technique that can detect glypican 3 in the range of 0.156-10 ng/ml and the sensitivity of the method to detect glypican 3 according to manufacturers is 0.014 ng/ml. The absorbance was measured at 450 nm.

Estimation of SGOT and SGPT

Estimation of SGOT/AST and SGPT/ALT was done through the biochemical Kinetics kit Method (CliniChem: USA-410S1) by spectrophotometer at wavelength 340 nm. The reference range according to the kit is 45 U/L.

Estimation of serum ALP

Alkaline phosphatase was measured using diethanolamine buffer, DJKC kit method. Reaction mixture with 20 ul test serum was monitored kinetically at 405 nm by the rate of formation of 4-nitrophenol, which is proportional to the presence of alkaline phosphatase present in the sample. The reference range for the determination of ALP according to the kit is 98-279 U/l.

Statistical analysis

The data was analyzed by a Statistical Program (SPSS). Student's-t test was applied to analyze levels of glypican 3, SGOT, SGPT and ALP. P<0.05 was taken as statistically significant.

Molecular docking

Molecular docking was performed using the empirical scoring function of Autodockvina. The empirical scoring function calculates the fitness of interaction by summing up the contribution of a number of individual turns, which is an important energetic factor in protein-ligand binding.

Preparation of ligand

Triptolide, compound ID 107985, was downloaded in SDF (2D) format from pub-Chem database. The structure of compound is shown in Figure 1.

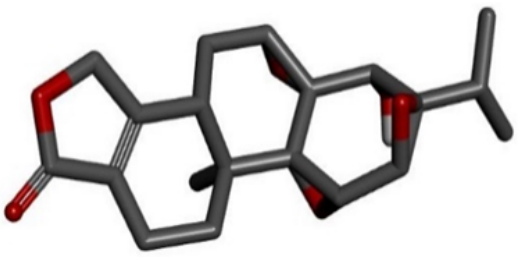

Figure 1: Ligand, Triptolide, and compound ID 107985.

Preparation of macromolecule

The 3D crystal structure of the glypican 3 (PDB ID; AF_AFP51654) was downloaded from the protein databank, RCSB (Research Collaboratory for Structural Bioinformatics) site in PDB Format (www.rcsb.org/pdb)

Setting docking parameter

Molecular docking was performed using Autodock 4.0, AutodockVina to predict maximum binding affinity of the ligands. The SDF (Spatial Data File) format of the ligands was converted into PDBQT, Protein (P), Data Bank (DB), partial charge (Q), and Atom type (T) format using command prompt from open babel for further docking studies. Macromolecule was saved in PDBQT format followed by deleting water molecule, addition of polar hydrogen and kolman charges in Autodock Tools (ADT). The docking parameter file was generated with grid spacing 1 Å and dimensions of 3.997 × 3.008 × 2.186 Å with x=50, y=50, and z=50 coordinates and saved as config_txt in the docking folder. The grids spacing used were set accordingly to keep enough space for ligand to be docked on the surface. Results were analyzed and a two-dimensional graphical depiction of best complex was assessed using discovery studio visualization.

RESULTS

Table 1 shows concentration of glypican 3 in both controls and samples and level of fluoride in serum and water collected from

Parameters	Controls from DHA	Test from narowal	t value
Fluoride in water (µM/L)	0.415 ± 0.3	0.575 ± 0.34	t=6.30 p=0.0001***
Fluoride in water (µM/L)	1.62 ± 0.24	4.60 ± 0.36	t=7.0318 p=0.0001***
Glypican3 (µM/L)	0.2 ± 0.0830	6.671 ± 0.790	t=5.3419 p=0.0001***

Note: Statistical analysis was performed using students-t test. All values are represented ad means +SEM. The significance of difference is indicated by ***P<0.0001when test group from DHA was compared with respective control group.

Table 1: Concentration of Glypican 3 and fluoride levels in serum and water in controls and samples.

the respective populations. Results analyzed by students- t test show significant increase in fluoride concentration in water t=6.30 (p=0.0001) and in blood t=7.0318 (p= 0.0001) of samples collected from Narowal when compared to controls. Further, glypican 3 t=5.3419 (p=0.0001) also shows marked increase in its concentration when compared to controls.

Table 2 shows different modes of ligand interaction with the receptors at various energy levels and Root Mean Square Deviation (RMSD). The table shows that lower root mean squares require higher binding energy. Table 3 shows amino acid moieties in binding pockets of glypican 3. Results show three pockets. Pocket 1 constitute LEU246, PHE248, SER249, GLY284, CYS 285, ALA287, GLY288, VAL289, LYS450, GLY 453, LEU 456, MET459 amino acids with the x=14.2065, y=-2.262 and z =-15.1986 axis; pocket 2 constitutes MET255, ARG258, TYR277, VAL281, MET441, PHE445, LEU447 amino acids with x=20.99 , y=0.09 and z =-26.3 axis; and pocket 3 constitutes MET255, TYR277, VAL280, VAL281, GLN434, ALA437, ARG438, MET441, LEU447 amino acids with the x=17.724, y=-2.104, and z=-29.26 axis.

Figure 2 shows significant increase in SGOT t=2.899 (p=0.004), SGPT 2.289 (p=0.02) and Alkaline Phosphatase t=6.29 (p=0.03) when compared to controls. Figure 3 shows docking pose generated by autodockVina with the highest binding energy -7.1 kcal/mol. Figure shows glutamine 231, serine 571 and aspargine 234 to be the amino acids involved in non-covalent interaction with the receptor, glypican3.

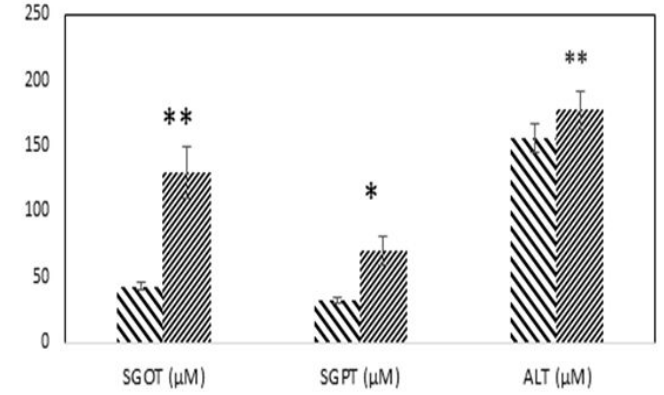

Figure 2: Statistical analysis was performed using student's t test. All values are expressed as means+SEM. The significance of difference is indicated by ***P<0.001,**P<0.01, *P<0.05 when test group was compared with respective control. **Note:** (▨) Controls, (▨) Sample.

Figure 4 shows docking pose of ligand interaction with receptor at highest binding energy (-7.1) showing conventional hydrogen bond interaction with serine 571 and aspargine 234 obtained through discovery studio. Figure shows vander Waal forces interaction with Gln 231, which is also confirmed by autodockvina with the highest binding energy with the ligand. In addition, amino acids Met 568, Val 572, Gln 112, Leu 157, Leu 235 and Glu 238 also show van der Waal interaction.

Mode	1	2	3	4	5	6	7	8	9
Binding affinity	-7.1	-6.9	-6.9	-6.9	-6.6	-6.3	-6.3	-6.2	-6.2
Rmsd	0.000	1.654	2.725	14.127	12.387	14.811	11.994	12.041	9.144

Table 2: Binding affinity of ligand with receptor at different energy levels and Root Mean Square Deviation (RMSD) values.

Active site pockets	No. of amino acid	Amino acid residues in active sites	Center x	Center y	Center z
Pocket 1	12	LEU246, PHE248, SER249, GLY284, CYS 285, ALA287, GLY288, VAL289, LYS450, GLY 453, LEU 456, MET459	14.2065	-2.262	-15.1986
Pocket 2	07	MET255, ARG258, TYR277, VAL281, MET441, PHE445, LEU447	20.9917	0.09	-26.3614
Pocket 3	09	MET255, TYR277, VAL280, VAL281, GLN434, ALA437, ARG438, MET441, LEU447	17.7242	-2.1044	-29.265

Table 3: Amino acid moieties in active site pockets of Glypican 3 with the x, y and z axis.

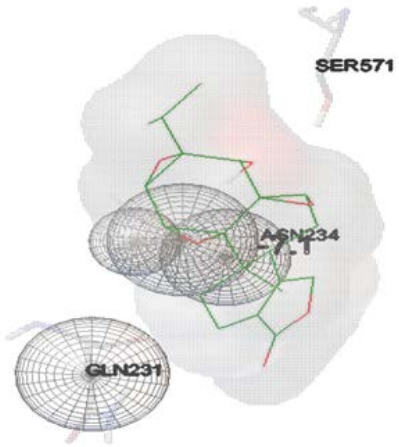

Figure 3: Docking pose generated by auto dock Vina with the highest binding energy -7.1 kcal/mol. Figure shows the glutamine 231, serine 571 and aspargine 234 are the amino acids involved in the non-covalent interaction with the receptor, glypican3.

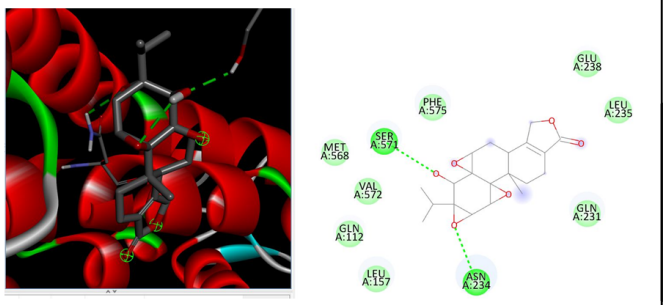

Figure 4: Binding of ligand with receptor at highest binding affinity showing non covalent interactions. **Note:** ( ) Hydrogen bonds and ( ) van der Waal forces.

DISCUSSION

Many natural processes and industrial activities cause contamination of ground water [29], resulting in fluoride contamination [30]. Present study shows that the rural area of district Narowal, Pakistan is an affected area of Pakistan with fluorotoxicity of drinking water. In parallel to present finding, Tharpakar, Peshawar, Nagar Parkar, Narowal, Dera Ghazi Khan, Lahore, Kasur, Faisalabad, Karachi, Sialkot have also reported flourotoxicity in drinking water [29,30-36]. These points to a serious challenge in healthcare for Pakistan-if this issue is not dealt with promptly, it could lead to life-threatening sequalae.

Management and controlling contamination by fluoride in drinking water is not an easy process and this toxicity is a threat to human health every day. High level of Fluoride content in the well-waters of district Narowal lead to significantly increased Fluoride levels in the serum of the consumers-this is well established in medical research and our study confirms that chronically elevated levels of fluoride in drinking water result in raised plasma fluoride levels [37].

Detection of glypican 3 levels in the plasma of the people of Narowal correlates their long-term exposure to fluoride in drinking water and the risk of developing HCC. Presence of *GPC3* in serum is a positive indicator of HCC [38,39] . Studies on tissue microarray and immunohistochemical data report that 70% of HCC samples show highly expressed *GPC3* compared to normal liver tissues, liver cirrhosis or hepatitis tissues, benign liver lesions [40]. Full-length glypican 3 is a better predictive biomarker than alpha-fetoprotein to assess risk of HCC recurrence after radial surgery [41]. Expression of *GPC3* is also correlated with poorer prognosis [42-45] further demonstrates the severity of the challenge Pakistan faces. On one hand, fluoridated water will not only lead to greater morbidity amongst the population, but it will also increase healthcare burden-with more cases of HCC emerging with time if this issue remains unresolved.

As stated earlier, people with a previously diagnosed cancer were excluded from this study. Hence, presence of HCC in serum of effected populations contributed to diagnosis. It can thus be suggested that long term consumption of fluoridated water played a major part in pathogenesis of the disease.

Prolonged fluoride exposure causes vacuolization of hepatocytes, dilated and hypertrophic liver tissue, cellular necrosis, oxidative stress, and cellular damage [46]. In the present study, fluoride exposure was associated with higher concentrations of plasma fluoride and altered liver enzymes. Raised SGOT and SGPT levels in the affected population point to a significant positive correlation between consuming high levels of fluoride and liver injury. This is supported by previous literature which shows histological alterations in the liver followed by exposure to high levels of fluoride [6]. A time course study on various concentrations of fluoride exposure to rats has been shown to alter liver proteins related to mitochondrial oxidative stress and endoplasmic reticulum [47]. Similar to our finding, a dose dependent study demonstrated that fluoride exposure greater than 2 mg/L may lead to alter kidney and liver enzymes function [48]. It is previously accepted in medical literature that overall survival and tumor free survival of patients with elevated SGOT and SGPT is far worse than those who have these enzymes in the normal ranges [49]. This further establishes risk for greater morbidity amongst HCC patients consuming fluoridated water

High levels of ALP in the people of Narowal consuming fluoridated water may also serve as a poor prognostic marker in HCC. ALP is a hydrolase enzyme that is present in all tissues of the entire body [47]. Clinically, it is used as a stable serum marker of biliary obstruction and related diseases. In addition, ALP is a potential prognostic marker in HCC and is included in the Chinese University Prognostic index [50]. Hence, levels of ALP could be used to assess therapeutic efficacy, in addition to liver function.

We have established so far that long-term consumption of fluoridated water contributes to liver damage, upregulation of Glypican-3 and possibly the development of HCC. Therefore it is of interest to see if HCC can be managed by inhibiting glypican3 at translational and transcriptional level.

Keeping in view the potential targets, present study investigated potency of triptolide to inhibit glypican 3 proteins by computational

docking studies. Binding of ligand to receptor at the lowest Root Mean Square Deviation (RMSD) (0.000) showed (Δ-7.1 kcal/mol) affinity by AutoDock Vina analysis. It was reported that if the RMSD values of the best conformation is<2.0 Å from the bound ligand in the experimental crystal structure then the used scoring function is successful. Results show few non-covalent interactions with glutamine 231, serine 571 and aspargine 234. Analysis of ligand and receptor interaction shows conventional hydrogen bond interaction with serine 571 and aspargine 234 while van der Waal forces interaction with Gln 231, Met 568, Val 572, Gln 112, Leu 157, Leu 235 and Glu 238 amino acids was observed. Triptolide exhibits a broad spectrum pharmacological profile, but it has also been reported for its narrow therapeutic window, poor water solubility (0.017 mg/ml, pH 7.4 at room temperature) and multi- organ toxicity including hepatotoxicity, nephrotoxicity, and reproductive toxicity [51-53].

Experimental evidences prove that triptolide induces apoptosis, suppresses inflammation and provokes cyto protection by inhibiting pro-inflammatory cytokines [54,55]. Active site analysis of glypican 3 shows three pockets where ligand can bind and contain 12, 7 and 9 amino acids in each pocket. Interaction of glypican 3 with triptolide according to present results obtained by autodock Vina does not show similarity to the amino acids present in the active site pockets-hence triptolide binds non-competitively at a site other than the active-site. In addition, Prank web analysis showing three different pockets for attachment indicate that the affinity of triptolide is not specific, rather, its binding outside the active site was observed at different x=3.997, y=3.008 and z=2.186 coordinates obtained by Auto dock Vina. This may represent its broad-spectrum mechanism of action-from its benefits to toxicity. Present results are in agreement with previous findings reporting toxicity and poor solubility in water, and due to this reason it is not used systemically in the clinic [56].

CONCLUSION

Therefore, triptolide analogues have been synthesized with diverse structural modifications. Structure activity relationship studies reported hydrogen bonded C-14 of triptolide performs a key role for its anticancer effects but its insolubility in water has delayed its use in the clinic. It is suggested that the high fluoride intake is one of the leading causes of HCC that can be diagnosed earlier with the detection of glypican 3. Its overexpression contributes to the development of HCC. Further, binding of triptolide to glypican3 in nonspecific manner demands structurally modified pharmacological targets for the prevention of hepatotoxicity and glypican-3 overexpression.

AUTHORS CONTRIBUTION

Iffat Ara (supervised and performed docking), Burhan Khatri (initial draft and revision of manuscript) Qurat-ul-Ain Chaudhary (data collection, hospital survey and laboratory estimations), Shakeel Ahmed (supervised data collection and laboratory estimations).

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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