Sorafenib induced alteration of protein glycosylation in hepatocellular carcinoma cells

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Abstract. Sorafenib is a multikinase inhibitor and is effective in treating hepatocellular carcinoma (HCC). However, it remains unknown whether sorafenib induces the alteration of protein glycosylation. The present study treated HCC MHCC97L and MHCC97H cells with a 50% inhibitory concentration of sorafenib. Following this treatment, alteration of protein glycosylation was detected using a lectin microarray. Compared with the controls, the binding capacity of glycoproteins extracted from sorafenib-treated HCC cells to the lectins *Bauhinia purpurea* lectin, *Dolichos biflorus* agglutinin, *Euonymus europaeus* lectin, *Helix aspersa* lectin, *Helix pomatia* lectin, Jacalin, *Maclura pomifera* lectin and *Vicia villosa* lectin were enhanced; while, the binding capacities to the lectins *Caragana arborescens* lectin, *Lycopersicon esculentum* lectin, *Limulus polyphemus* lectin, *Maackia amurensis* lecin I, *Phaseolus vulgaris* leucoagglutinin, *Ricinus communis* agglutinin 60, *Sambucus nigra* lectin and *Solanum tuberosum* lectin were reduced (spot intensity median/background intensity median ≥2, P<0.05). This difference in glycoprotein binding capacity indicates that cells treated with sorafenib could increase α-1,3GalNAc/Gal, β-1,3 Gal, GalNAcα-Ser/Thr(Tn) and α-GalNAc structures and decrease GlcNAc, sialic acid, tetra-antennary complex-type N-glycan and β-1,4Gal structures. These results were additionally confirmed by lectin blotting. Expression levels of signaling molecules including erythroblastosis 26-1 (Ets-1), extracellular signal-related kinases (ERK) and phosphorylated-ERK were measured by western blotting. There was a reduction in the expression of Ets-1 and ERK phosphorylation in sorafenib or 1,4-Diamino-2,3-dicyano-1,4-bis (2-aminophenylthio) butadiene treated cells suggesting that sorafenib may reduce the expression levels of Ets-1 by blocking the Ras/Raf/mitogen activated protein kinase signaling pathway. In the present study, it was clear that sorafenib could inhibit the proliferation of HCC cells and alter protein glycosylation. The findings of this study may lead to providing a novel way of designing new anti-HCC drugs.

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Introduction

Hepatocellular carcinoma (HCC) is the sixth most common type of cancer in the world, and has received considerable attention in previous years (1). The occurrence and development of HCC is a result of a multiple gene regulation cascade (2,3). During a multiple gene regulation cascade, there is often an alteration of protein glycosylation (4). Glycosylation is one of the most important protein post-translational modifications, and >50% of proteins in nature are presumed to have undergone glycosylation. Glycosylation performs a key role in controlling various cellular processes, including cellular adhesion, receptor activation, signal transduction and endocytosis (5). Alteration of glycosylation can be observed in numerous diseases and some of these alterations are well-known biomarkers in cancer progression (6,7). During the occurrence and development of HCC, glycan branched structures such as GlcNAc-branched N-glycans, sialic acid and multi-antennary glycans with fucose residues are highly expressed (8,9).

Sorafenib, which has been approved by the U.S. Food and Drug Administration and the European Medicine Agency, is a multikinase inhibitor (10). Growing evidence suggests that sorafenib has shown efficacy against a wide variety of tumors such as advanced renal cell carcinoma and HCC (11). Sorafenib has the following possible targets: Raf; epidermal growth factor receptor; vascular endothelial growth factor receptor; FMS-like tyrosine kinase-3; platelet-derived growth factor receptor; and c-kit, and may block cellular proliferation and angiogenesis (12). Sorafenib, as a molecular targeted drug against HCC, has increased effectiveness and fewer adverse reactions in comparison with other HCC chemotherapy drugs, including Adriamycin (13). As an intracellular signaling pathway blocking agent, a number of studies have explored the exact molecular mechanism of how sorafenib works (14-16). However, there have been few studies into the

sorafenib-induced alteration of protein glycosylation. If more attention was paid to the alteration of protein glycosylation in sorafenib-treated HCC cells, novel and interesting findings may be identified.

With the development of glycan-captured techniques, the lectin microarray is widely used for glycoproteomics and glycomics studies (17,18). In previous studies, we designed and established a lectin microarray with 50 different lectins (19,20). To detect the effect of sorafenib on the alteration of protein glycosylation, the present study compared the glycosylation profiles of HCC cells with or without sorafenib treatment using a lectin microarray and found that expression levels of 2 tumor metastasis-associated glycan structures, sialic acid and tetra-antennary complex-type N-glycan, were decreased.

The present study also compared expression levels of signaling molecules such as erythroblastosis 26-1 (Ets-1), which takes part in tumor invasion, metastasis and glycosylation. To the best of our knowledge, the present study is the first to report that sorafenib treatment could reduce the expression of Ets-1.

Materials and methods

Cell cultures and treatment. Human HCC MHCC97L and MHCC97H cell lines have the same genetic background and were established in the Liver Cancer Institute of Fudan University (Shanghai, China) (21). All cells were cultured in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc.) and were incubated at 37˚C with 5% CO_2. Sorafenib was provided by Bayer HealthCare Pharmaceuticals Inc. (Whippany, NJ, USA) and was solubilized using dimethyl sulfoxide (DMSO; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Subsequent to cells being seeded for 24 h, sorafenib-treated groups were supplemented with the 50% inhibitory concentration (IC_{50}) of sorafenib (MHCC97L, 14.18 μmol/l; MHCC97H, 15.08 μmol/l). A 0.1% solution of DMSO (vol/vol) was also added to culture medium to serve as the control group, and the two treatments were then incubated at 37˚C for a further 24 h. In order to investigate the effect on expression levels of Ets-1 by targeting the Ras/Raf/mitogen-activated protein kinase (MAPK) signaling pathway, HCC MHCC97L and MHCC97H cells were incubated with or without U0126 (20 μmol/l; Selleck Chemicals, Houston, TX, USA) for 48 h.

Cell proliferation assay. The Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Kyushu, Japan) assay was used to determine the relative growth of cell groups with or without sorafenib treatment. MHCC97L and MHCC97H ($3x10^3$ cells/well) were seeded into 96-well plates. Subsequent to a 24 h incubation at 37˚C, cells were incubated with distinct sorafenib concentrations (0, 4, 8, 12, 16, 20, 24, 28 and 32 μmol/l) and the experiment was performed three times at each concentration. DMSO was added to cultures at 0.1% (v/v) as solvent controls, all cells were incubated at 37˚C for another 24 h. Cells were then stained with 10 μl CCK-8 solution for 1 h at 37˚C. Absorbance was measured at 450 nm using the microplate reader, Infinite 200 PRO NanoQuant (Tecan, Männedorf, Switzerland). Each treatment was repeated in 5 wells and the IC_{50} values were calculated from growth inhibition curves.

Protein extraction. Cells were rinsed with PBS (Thermo Fisher Scientific, Inc.) 3 times and lysed with radioimmunoprecipitation assay lysis buffer (Thermo Fisher Scientific, Inc.) containing an complete EDTA-free protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). Protein concentrations were measured with a Pierce BCA Protein assay kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol, and used for additional experiments as described below.

Lectin microarray. Total extracted proteins from the cells were exchanged in PBS by PD-10 desalting columns (GE Healthcare, Piscataway, NJ, USA) and were biotinylated by the Lightning-Link Biotin Labeling kit according to the manufacturer's protocol (Innova Biosciences, Cambridge, UK). The lectin microarray with 50 different types of lectins, 2 CY3 positive controls and 2 blank controls (Fig. 1A) was used according to the following protocol: The lectin microarray covered with 2% bovine serum albumin (BSA; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) in PBS solution at room temperature for 1 h to block the non-specific binding sites, followed by washing with 0.1% TBS-Tween-20 (TBST; 50 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.6) for 5 min 3 times. Biotinylated proteins were then loaded (100 μl/block) and incubated at room temperature with gentle agitation for 3 h. The lectin microarray was then washed with 0.1% TBST for 5 min 3 times. Subsequently, the CY5 labeled streptavidin (Thermo Fisher Scientific, Inc.) was loaded into each block and incubated for 1 h at room temperature with gentle agitation. Subsequent to washing with 0.1% TBST for 5 min 3 times, the lectin microarray was scanned using the LuxScan 10K/A scanner system (CapitalBio, Beijing, China).

Lectin microarray data were analyzed as previously described (12). Briefly, the spot intensity median (S), which was the lectin spot-glycoprotein binding intensity median, and the background intensity median (B) were extracted by Luxscan 3.0 (CapitalBio, Beijing, China). The S to B (S/B) of each lectin spot and average S/B of 6 replicate spots for each lectin in every block were calculated. MeV 4.8.1 software (Dana-Farber Cancer Institute, Boston, MA, USA) was used to map hierarchical clustering of S/B of lectins with the same tendency in the two kinds of sorafenib-treated cells compared with control groups. S/B ≥2 was set as the cut off and spots with S/B ≥2 were termed the positive lectin binding spots.

Lectin blotting. Equal proteins extracted from cells were additionally separated by 10% SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes (Merck Millipore). Membranes were blocked for nonspecific binding with 3% BSA-TBST at room temperature for 1 h and then incubated with biotinylated *Maackia amurensis* lectin I (MAL-I; catalog no., B-1315; Vector Laboratories, Inc., Burlingame, CA, USA), biotinylated leucoagglutinin (PHA-L; catalog no., B-1115; Vector Laboratories, Inc.) at a dilution of 1:2,000 or a primary monoclonal mouse anti-human β-actin antibody (cat.

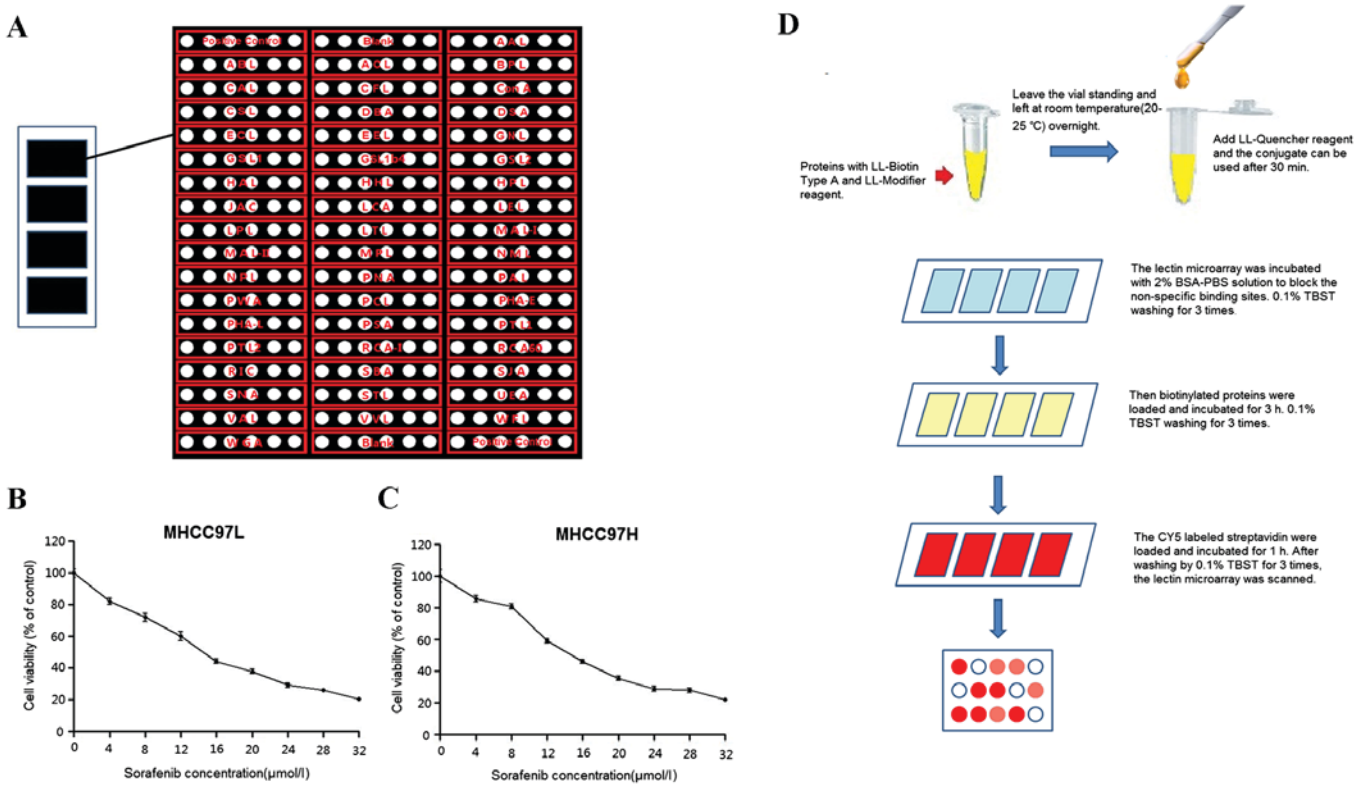

Figure 1. Lectin microarray system and effect of Sorafenib on cell viability of HCC cells. (A) The layout of the lectin microarray: The lectin microarray consists of 50 different specific binding lectins, 2 positive controls and 2 blank controls, each lectin was repeated for 6 spots in one block. (B) Cell viability of MHCC97L cells incubated with Sorafenib (0-32 μmol/l) for 24 h. (C) Cell viability of MHCC97H cells incubated with Sorafenib (0-32 μmol/l) for 24 h. (D) The workflow of profiling glycosylation of proteins extracted from HCC cells by the lectin microarray. Data represented as the mean ± the standard deviation. HCC, hepatocellular carcinoma; BSA-PBS, bovine serum albumin-PBS; TBST, TBS and Tween-20.

no. KC-5A08; KangCheng Bio-tech, Shanghai, China) at a dilution of 1:2,000 at room temperature for 30 min. Subsequent to washing with 0.1% TBST for 10 min 3 times, membranes were incubated with streptavidin horseradish peroxidase (HRP) conjugate (Invitrogen; Thermo Fisher Scientific, Inc.) at a dilution of 1:10,000 or HRP-conjugated secondary antibody (cat. no. KC-MM-035; KangCheng Bio-tech) at a dilution of 1:10,000 at room temperature for another 30 min, and washed by 0.1% TBST for 10 min 3 times. The bands on the membranes were detected by using Amersham enhanced chemiluminescence (ECL) prime western blotting detection reagents (GE Healthcare). Anti-β-actin antibody was used as the protein loading control.

Western blotting. Equal proteins were separated using 10% SDS-PAGE and transferred to PVDF membranes. Subsequent to blocking with 5% milk-TBST at room temperature for 1 h, membranes were incubated with 4 different antibodies followed by an incubation at 4˚C overnight. The antibodies used are as follows: Polyclonal rabbit anti-human Ets-1 antibody (dilution, 1:1,000; cat. no. 6258; Cell Signaling Technology, Danvers, MA, USA); monoclonal rabbit anti-human extracellular signal-related kinase (ERK) antibody (dilution, 1:2,000; cat. no. 4695; Cell Signaling Technology); monoclonal rabbit anti-human phospho-ERK antibody (p-ERK; dilution, 1:2,000; cat. no. 4370; Cell Signaling Technology); and a monoclonal mouse anti-human β-actin antibody (dilution, 1:2,000). Membranes were washed using 0.1% TBST for 10 min 3 times and incubated in HRP-conjugated secondary polyclonal antibodies (dilution, 1:10,000; KC-MM-035 and KC-RB-035; KangCheng Bio-tech) at room temperature for 1 h, and washed using 0.1% TBST for 10 min 3 times. Amersham ECL detection (GE Healthcare) was used to visualize the bands on membranes and β-actin was detected using the anti-β-actin antibody that was used as the loading control for proteins. Quantitative analysis of protein bands was performed using Quantity One software (version 4.62; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. IC_{50} values were calculated using GraphPad Prism version 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). Statistical analyses were performed using SPSS 16.0 statistical packages (SPSS Inc., Chicago, IL, USA). Quantitative variables were analyzed using the Student's *t*-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Inhibitory effect on cell proliferation in sorafenib-treated HCC cells. The present study treated MHCC97L and MHCC97H cells with several different doses of sorafenib: 0, 4, 8, 12, 16, 20, 24, 28 and 32 μmol/l for 24 h to determine the IC_{50}. Proliferation of MHCC97L and MHCC97H cells was inhibited following sorafenib treatment and the IC_{50} values were 14.18 and 15.08 μmol/l, respectively (Fig. 1B and C). The present study chose to treat MHCC97L and MHCC97H cells

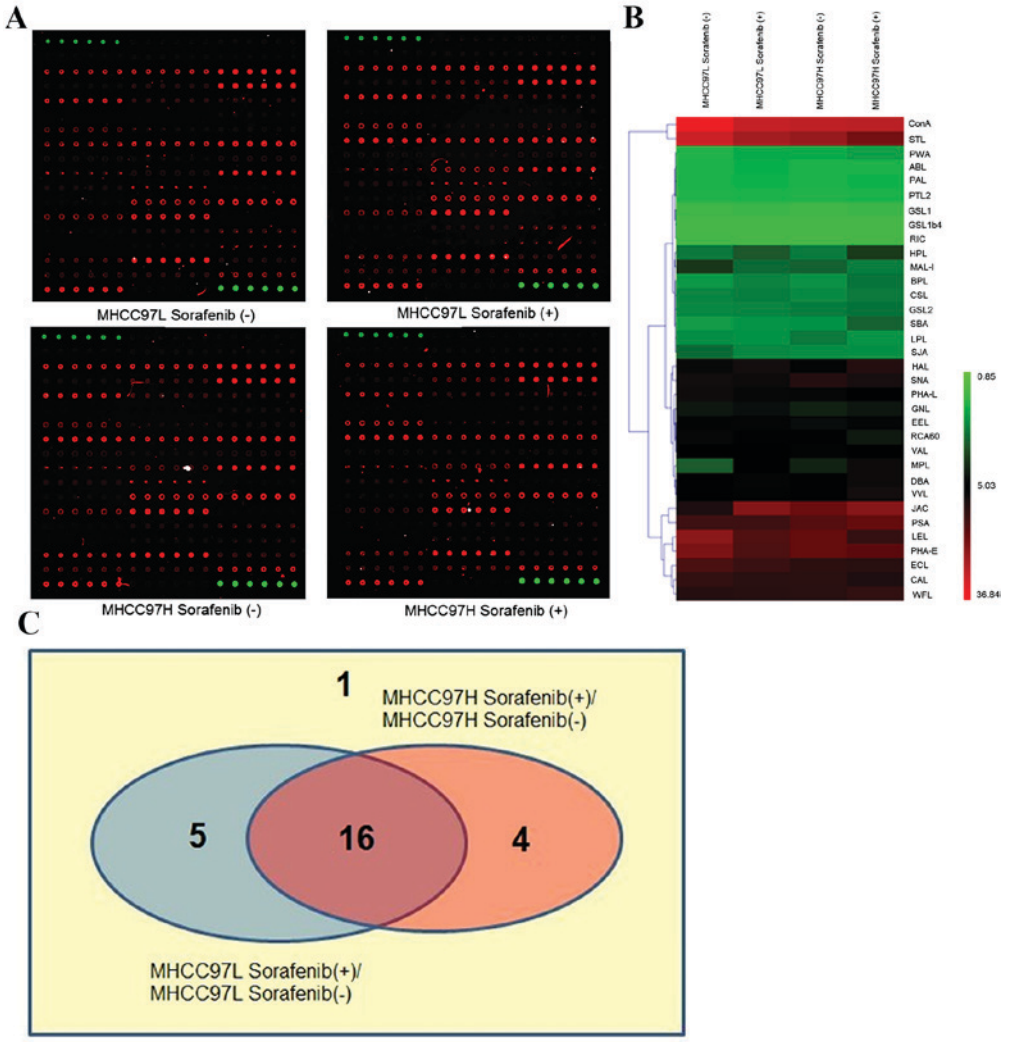

Figure 2. Results of differential glycosylation profiles of proteins extracted from HCC cells with or without sorafenib treatment by the lectin microarray. (A) Lectin microarray was scanned by LuxScan 10K/A scanner system. Positive controls are shown on the left and representative images of proteins extracted from HCC cells with or without sorafenib treatment binding with lectin microarray are shown on the right. (B) Hierarchical clustering of the lectin-proteins binding profiles by using the lectins revealed the same S/B trend, each row represented a lectin. Red represents a lectin with a high S/B trend value and green represents a lectin with a low S/B value. (C) A Venn diagram of positive lectin binding spots demonstrates lectins with the common and unique binding capacity to proteins in MHCC97L and MHCC97H cell with or without sorafenib treatment. The numbers refer to the count of lectins identified in each group: 1, the VAL whose binding capacity was unchanged in the two types of sorafenib-treated HCC cells; 4, the lectins whose binding capacities were significantly different in sorafenib-treated MHCC97H cells (but not significant in sorafenib-treated MHCC97L cells); 5, the lectins that altered in the sorafenib-treated MHCC97L cells 9 (but their binding capacities of proteins in sorafenib-treated MHCC97H cells were unaltered); and 16, the lectins that were altered significantly in the two sorafenib-treated HCC cells compared with the control groups. S/B, spot intensity median/background intensity median. HCC, hepatocellular carcinoma; VAL, viscum album lectin.

with IC_{50} to additionally investigate the glycosylation profiles of HCC cells following sorafenib treatment.

Alteration of protein glycosylation in sorafenib-treated HCC cells. Proteins extracted from sorafenib-treated groups and control groups were applied to the lectin microarray, including 50 lectins, 2 CY3 positive controls and 2 blank controls for each block. The workflow is shown in Fig. 1D and lectin microarrays were scanned by a LuxScan 10K/A scanner system. The data obtained indicated that the protein glycosylation profiles had been changed in sorafenib-treated HCC cells compared with the control groups (Fig. 2A). The binding capacities of 34 lectins had the same tendency in the two types of sorafenib-treated cells compared with control groups, in which 19 were upregulated while 15 were downregulated. The hierarchical clustering of S/B of these lectins was mapped using the MeV 4.8.1 software (Fig. 2B).

Among these 34 lectins, S/B of 26 lectins was ≥2 and these were defined as positive lectin binding spots (Fig. 2C). Viscum album lectin, shown as '1' in the Venn diagram, was the lectin whose binding capacity was unchanged in the two types of sorafenib-treated HCC cells compared with control groups (MHCC97L, P=0.939; MHCC97H, P=0.389). Concanavalin A, *Erythrina crista-galli* lectin, *Phaseolus vulgaris* Erythroagglutinin, *Pisum sativum* agglutinin and *Sophora japonica* agglutinin, are shown as '5' in the Venn diagram, and were lectins with P<0.05 in sorafenib-treated MHCC97L cells compared with untreated MHCC97L cells, but their binding capacities of proteins in sorafenib-treated MHCC97H cells were unchanged (P≥0.05). In contrast to

Table I. Lectins with changed binding capacities and their specific binding abilities.

Abbreviation	Lectin	Specificity	Monosaccharide specificity
BPL	*Bauhinia purpurea* lectin	Galβ1-3GalNAc	Galactose
CAL	*Caragana arborescens* lectin	N-Acetylgalactosamine	GalNAc
DBA	*Dolichos biflorus* agglutinin	GalNAcα1-3Gal; GalNAcα-Ser/Thr(Tn)	GalNAc
EEL	*Euonymus europaeus* lectin	Galα1-3(Fucα1-2)Gal	Galactose
HAL	*Helix aspersa* lectin	terminal N-acetyl-α-D-galactosaminyl residues	αGalNAc
HPL	*Helix pomatia* lectin	α-N-acetyl-D-galactosamine	αGalNAc
JAC	Jacalin	Galβ1-3GalNAcα-Ser/Thr(T); GalNAcα-Ser/Thr(T)	Galactose; GalNAc
LEL	*Lycopersicon esculentum* lectin	Ploy-LacNAc; (GlcNAc)n	LacNAc; GlcNAc
LPL	*Limulus polyphemus* lectin	N-acetyl-D-hexosamines	HexNac
MAL-I	*Maackia amurensis* lecin I	gal (β-1,4) glcNAc; Siaα2-3Gal	Galactose; Sialic acid
MPL	*Maclura pomifera* lectin	αGalNAc T antigen/Tn antigen	αGalNAc
PHA-L	*Phaseolus vulgaris* leucoagglutinin	Tetraantennary complex-type N-glycan	Complex
RCA60	*Ricinus communis* agglutinin 60	galactose; N-Acetylgalactosamine	Galactose; GalNAc
SNA	*Sambucus nigra* lectin	Sia2-6Galβ1-4GlcNAc	Sialic acid
STL	*Solanum tuberosum* lectin	(GlcNAc)n	GlcNAc
VVL	*Vicia villosa* lectin	N-Acetylgalactosamine; GalNAcα-Ser/Thr(Tn)	GalNAc

the untreated MHCC97H cells, *Cytisus scoparius* lectin, *Galanthus nivalis* lectin, *Griffonia simplicifolia* lectin II and wheat germ agglutinin, (shown as '4' in the Venn diagram), were lectins whose binding capacities were significantly different in sorafenib-treated MHCC97H cells ($P<0.05$), but were not significant ($P\geq0.05$) in sorafenib-treated MHCC97L cells compared with the control group. Finally, the binding capacities of 16 letins: *Bauhinia purpurea* lectin (BPL), *Caragana arborescens* lectin (CAL), *Dolichos biflorus* agglutinin (DBA), *Euonymus europaeus* lectin (EEL), *Helix aspersa* lectin (HAL), *Helix pomatia* lectin (HPL), Jacalin (JAC), *Lycopersicon esculentum* lectin (LEL), *Limulus polyphemus* lectin (LPL), MAL-I, *Maclura pomifera* lectin (MPL), PHA-L, *Ricinus communis* agglutinin 60 (RCA60), *Sambucus nigra* lectin (SNA), *Solanum tuberosum* lectin (STL) and *Vicia villosa* lectin (VVL), shown as '16' in the Venn diagram, were changed significantly in the two sorafenib-treated HCC cells compared with control groups (all $P<0.05$). Their full names and specificities were listed in Table I.

Fig. 3 showed specific binding abilities and quantitative results of S/B of the aforementioned 16 lectins. Binding capacities of 8 lectins were upregulated in sorafenib-treated cells comparing with control groups (Fig. 3A). This group included β-1,3Gal binder BPL, Galβ1-3GalNAcα-Ser/Thr(T) and GalNAcα-Ser/Thr(Tn) binder JAC, α-1,3 Gal binder EEL, α-1,3GalNAc and GalNAcα-Ser/Thr(Tn) binder DBA, GalNAc and GalNAcα-Ser/Thr(Tn) binder VVL, α-GalNAc binders HAL, HPL and MPL. By contrast, the binding capacities of 8 lectins were reduced compared with the controls; this group of lectins included α-2,3Sia and β-1,4Gal binder MAL-I, α-2,6Sia binder SNA, GlcNAc and Ploy-LacNAc binder LEL, GlcNAc binder STL, GlcNAc and GalNAc binder LPL, tetra-antennary complex-type N-glycan binder PHA-L, Gal and GalNAc binder RCA60, GalNAc binder CAL (Fig. 3B). According to these results, sorafenib treatment could increase binding capacities of glycoproteins to α-1,3GalNAc/Gal, β-1,3Gal, GalNAcα-Ser/Thr(Tn) and αGalNAc binder lectins, but decrease binding capacities to GlcNAc, sialic acid, tetra-antennary complex-type N-glycan and β-1,4Gal binder lectins in MHCC97L and MHCC97H cells.

Validation of protein glycosylation alteration. To validate these results and provide increased evidence, lectin blotting was performed using the biotinylated MAL-I, biotinylated PHA-L lectins. Results are shown in Fig. 4A and additionally confirmed that sorafenib treatment could reduce sialic acid and tetra-antennary complex-type N-glycan in HCC cells.

Sorafenib inhibited the Ras/Raf/MAPK signaling pathway and reduced the expression of Ets-1. To determine whether sorafenib could affect the expression of Ets-1, the present study measured expression levels of Ets-1, ERK and p-ERK by western blotting. As presented in Fig. 4B, expression levels of ERK were not apparently altered in sorafenib-treated MHCC97L and MHCC97H HCC cells. However, the relative band intensity (sorafenib-treated/without sorafenib-treated) showed that the expression levels of ERK and Ets-1 phosphorylation were significantly reduced following sorafenib treatment in both cell lines compared with the control (all $P<0.05$). The same results could be observed in HCC MHCC97L and MHCC97H cells treated with 1,4-Diamino-2,3-dicyano-1,4-bis (2-aminophenylthio) butadiene (U0126), an inhibitor on kinase activity of MEK1/2 (Fig. 4C).

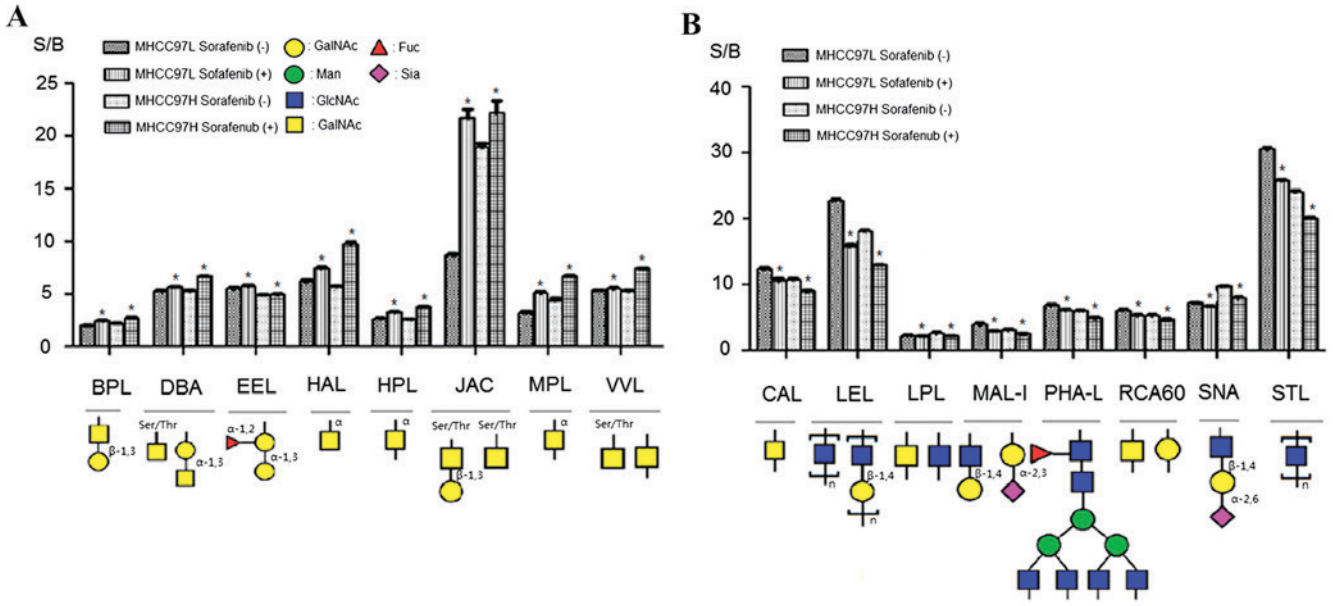

Figure 3. Specific binding abilities and quantitative results of positive lectin binding spots (S/B ≥2, P<0.05) from lectin microarray. (A) Lectins with upregulated binding capacity of glycoproteins in Sorafenib-treated cell. (B) Lectins with downregulated binding capacity of glycoproteins in Sorafenib-treated cell. Data is presented as the mean ± standard deviation. °P<0.05.

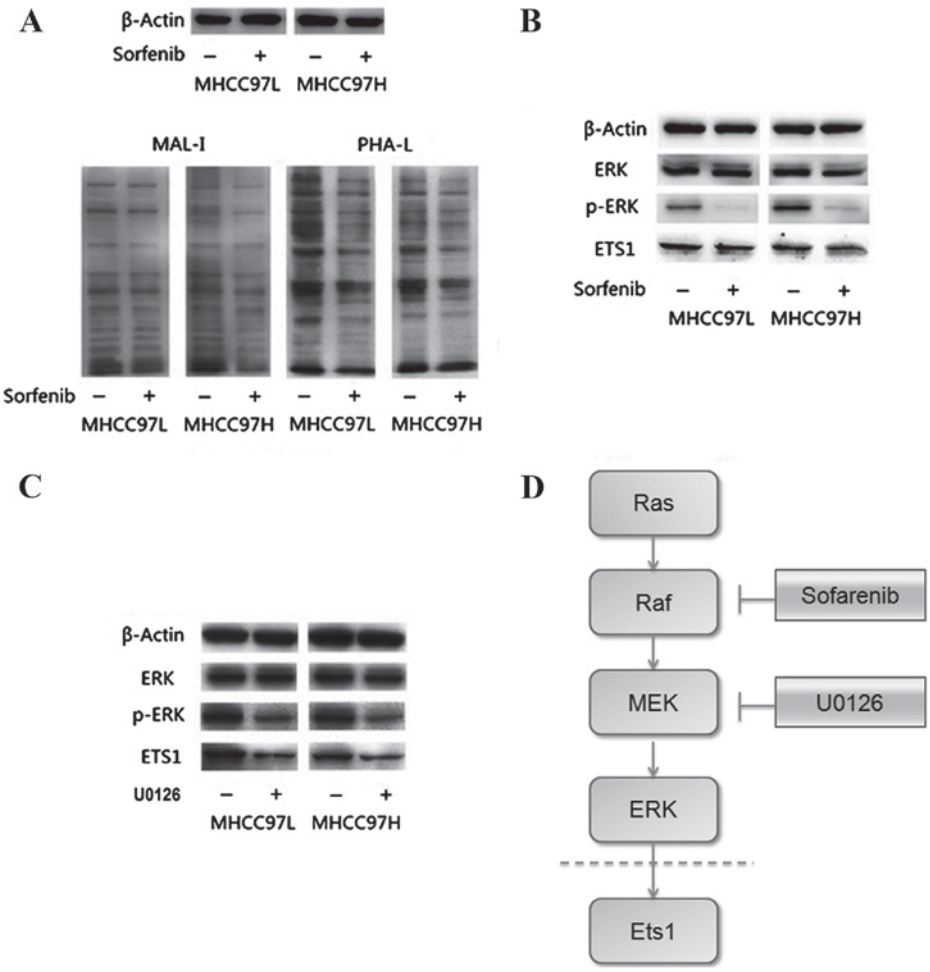

Figure 4. Results of lectin blotting and western blotting. (A) Lectin blotting validated Sorafenib induced alteration by the biotinylated MAL-I and biotinylated PHA-L. Anti-β-actin antibody was used to show the protein loading. (B) Downregulation of p-ERK in sorafenib-treated MHCC97L and MHCC97H cells. (C) Downregulation of p-ERK in MHCC97L and MHCC97H cells with U0126 treatment. (D) Diagram of the possible mechanism by which sorafenib treatment reduced the expression of Ets-1. sorafenib treatment decreased the expression of Ets-1 by blocking the Ras/Raf/MAPK signaling pathway in HCC cells. MAL-1, *Maackia amurensis* lecin I; PHA-L, Phaseolus vulgaris leucoagglutinin; p-, phosphorylated; MEK, mitogen activated protein kinase; ERK, extracellular signal-related kinases; Ets-1, erythroblastosis-26; U0126, 1,4-Diamino-2,3-dicyano-1,4-bis (2-aminophenylthio) butadiene.

Discussion

There are 5 types of glycosylation: N-linked glycosylation; O-linked glycosylation; C-glycosylation; phosphoglycosylation and glypiation (GPI anchor attachment). Glycosylation is an important protein posttranslational modification and regulates numerous critical cellular processes. Blomme *et al* (22) revealed that in association with the development of HCC, the N-glycosylation of serum proteins was modified and the branching was increased. The study by Dennis *et al* (23) has shown that high branching of N-linked glycans appears to be associated with the development of malignancy. Shao *et al* (24) has suggested that an increased formation of tetra-antennary complex-type N-glycan may contribute to the malignant and metastatic potential of tumor cells. A recent study revealed that sialylation may regulate the invasion and chemosensitivity of HCC by regulating the activity of the phosphoinositol 3-kinase/protein kinase B signaling pathway (25).

The present study demonstrated that glycosylation was altered in sorafenib-treated HCC cells. Glycosylation profiles were analyzed in a rapid, sensitive and high-throughput manner using the lectin microarray. In contrast to the control groups, glycoproteins extracted from sorafenib-treated HCC cells had altered binding capacities to 16 lectins ($S/B \geq 2$, $P<0.05$). Previous studies regarding sorafenib have mostly focused on discovering associated signaling molecules as potential targets for the therapy of HCC. To the best of our knowledge, the present study is the first report on the effect of sorafenib on glycosylation profiles. The present study demonstrated that the binding capacities of proteins extracted from sorafenib treated HCC cells to α-1,3GalNAc/Gal, β-1,3Gal, GalNAcα-Ser/Thr(Tn) and αGalNAc binder lectins were upregulated, while binding capacities to GlcNAc, sialic acid, tetra-antennary complex-type N-glycan and β-1,4Gal binder lectins were downregulated. These results suggest that sorafenib may inhibit the progression of HCC by affecting chain structures of glycoproteins. In addition, this finding provides a novel way for designing new anti-HCC drugs by anti-abnormal glycosylation.

In the HCC model PLC/PRF/5, sorafenib could block the Ras/Raf/MAPK signaling pathway (12), and the present western blotting results were consistent with this finding. Furthermore, it was first identified and confirmed that the expression of Ets-1, which is associated with tumor angiogenesis and tumor invasion, was reduced following the blocking of the Ras/Raf/MAPK signaling pathway with sorafenib or U0126 (Fig. 4D). Circumstantial evidence from an earlier study led the authors to consider that blocking the Ras/Raf/MAPK signaling pathway may affect the activation of vascular endothelial growth factor by inhibited ERK phosphorylation, which then leads to a reduced expression of Ets-1 (26). The expression levels of downstream Ets-1 in the Ras/Raf/MAPK signaling path way exhibited a pattern similar to GnT-V expression in a variety of HCC cell lines, including HuH7, Hep3B and HepG2, and Ets-1 would be expected to promote tumor metastasis by enhancing the expression of UDP-N-acetylglucosamine:α-6-D-manno-side β1-6-N-acetylglucosaminyltransferase (GnT-V; enzyme accession number, EC2.4.1.155) (27). Glycans are catalyzed by glycosyltransferases. The concept of one enzyme-one linkage describes that glycosyltransferases are specific for a single glycosyl donor, acceptor or alteration in transcription (28,29). Changes in expression levels of glycosyltransferases are associated with abnormal glycosylation (30). GnT-V located in the golgi apparatus synthesizes tetra-antennary complex-type N-glycan and the increased tetra-antennary complex-type N-glycan is linked to increased tumor metastasis (31,32). The gene Mgat5 codes for GnT-V and there are 3 Ets binding sites located in the 5' flanking region of Mgat5 and expression levels of Ets-1 and GnT-V are similar in human hepatoma tissues (27). The present results highlighted that tetra-antennary complex-type N-glycan was reduced in sorafenib-treated HCC cells. However, additional studies are required to determine whether sorafenib reduces the tetra-antennary complex-type N-glycan by affecting the expression of GnT-V by blocking the Ras/Raf/MAPK signaling pathway. In addition, influences of altered glycosylation by sorafenib treatment to HCC cells biological behavior may be worthy of additional investigation.

In conclusion, the present findings indicate that sorafenib could inhibit proliferation of HCC cells and in sorafenib-treated HCC cells glycan structures α-1,3GalNAc/Gal, β-1,3Gal, GalNAcα-Ser/Thr(Tn) and α-GalNAc were highly expressed, while GlcNAc, sialic acid, tetra-antennary complex-type N-glycan and β-1,4Gal had low expression levels. Furthermore, the present study is the first to identify that sorafenib reduces the expression of Ets-1, which was associated with the glycosylation of proteins, by blocking the Ras/Raf/MAPK signaling pathway. The present findings may therefore provide research to identify new anti-HCC drugs.

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