Genes & Diseases (2022) 9, 579–581

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RAPID COMMUNICATION

Sex hormone-binding globulin impedes hepatocellular carcinoma growth via MERTK regulation

Sex hormone-binding globulin (SHBG), a crucial modulator of sex steroids in human blood, regulates androgen bioavailability and homeostasis.[1] When androgen or AR signaling is known to be involved in the hepatocellular carcinoma (HCC) and SHBG plays an integral role in regulating the levels of bioavailable androgens, the concern whether SHBG might influence HCC onset or progression by modulating androgen action remains open. Our study aims to investigate the role of SHBG in HCC progression in contexts of androgen regulation and steroid independence.

In clinical data, *SHBG* mRNA expression in tumors was significantly lower than adjacent non-tumor liver tissues (Fig. 1A, S1A), and was significantly lower in HCC patients compared to non-HCC patients (Fig. S1B). Interestingly, long tumor doubling time and small tumor size were observed in HCC patients possessing high *SHBG* expression (Fig. S1C,D). Immunofluorescent SHBG protein level was markedly suppressed in HCC grade 4 (Fig. S1E). Furthermore, overall survival duration was significantly extended in HCC patients with high *SHBG* expression (Fig. 1B, S1F, G). It also should be noted that patients expressing tumor SHBG levels similar to those of NT have a better prognosis. To investigate how SHBG affects HCC, we induced HCC in 2-week-old male mice expressing human full-length *SHBG* gene (*SHBG*-Tg) and evaluated the tumor progression at 40-week-old. Representative gross images and H&E staining of tumors were associated with reduced the number of tumor nodules, as evidenced by the sum and average in size of tumor nodules (Fig. 1C–E, S2A). Consistent with clinical data, *SHBG* mRNA level in the tumor was lower than that in the normal liver parenchyma (Fig. S2B), but SHBG protein was significantly concentrated in the HCC region (Fig. S2C, D). Likewise, acute diethylnitrosamine (200 mg/kg) administration to *SHBG*-mice or *SHBG*-administered mice showed a similar *SHBG* expression tendency (Fig. S3, 4). Since SHBG internalization and SHBG receptor are not clear, we hypothesized that extracellular-accumulated SHBG exists and would play a role in HCC regulation.

The plasma of *SHBG*-Tg mice significantly decreased HMGB1 level, which represents necrotic DAMPs (Fig. 1F). Conversely, tumors from *SHBG*-Tg mice showed significantly higher apoptotic gene levels and TUNEL positive signals, and had lower PCNA levels and proliferation-related transcripts (Fig. 1G–K). As apoptosis minimizes inflammation while necrosis produces inflammatory content, proliferation rates of *SHBG*-tumor should be affected by inflammation. Indeed, IL-1β levels and pro-inflammatory mRNA levels were suppressed in tumor-bearing *SHBG*-Tg mice (Fig. 1L, S5A). Furthermore, *SHBG*-Tg mice decreased levels of fibrotic markers, invasiveness markers, and C-MET signaling proteins which are related to inflammation (Fig. S5B–D). Through presented results, we inferred that SHBG suppresses DAMPs production from necrotic hepatocytes and hinders pro-inflammatory response from Kupffer cells. Though SHBG regulates androgen bioavailability, pro-inflammatory cytokines were not markedly affected by testosterone in Raw 264.7 cells (Fig. S6). However, Raw 264.7 cell activation with high IL-1β were substantially suppressed in SHBG plasma or pure SHBG-treated group (Fig. 1M, N, S7), suggesting SHBG could play as a signal-modulator in HCC progression regardless of testosterone engagement.

Along with increased HCC risk in androgenic females,[2] testosterone withdrawal in WT and *SHBG*-Tg mice decreased HCC progression (Fig. S8A). When plasma testosterone levels were substantially higher in *SHBG*-Tg mice than in WT mice at a 40-week-old (Fig. 1O), the AR level and AR target gene levels were suppressed in SHBG-tumor (Fig. 1P, S8B). Like mouse model, *SHBG*

https://doi.org/10.1016/j.gendis.2021.10.003

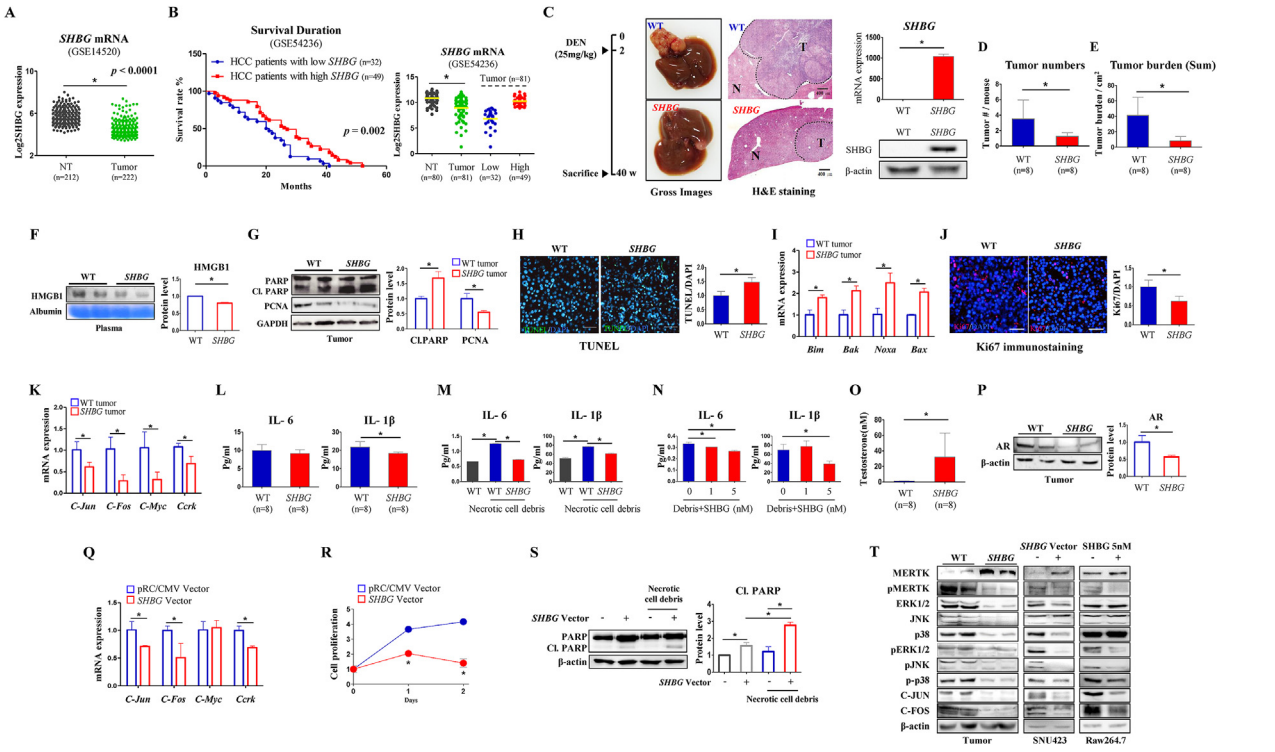

Figure 1 SHBG decreases HCC growth by suppressing cancer cell proliferation and pro-inflammatory responses via MERTK regulation. (A) Scatter plots of *SHBG* transcription levels in HCC-bearing patients (GSE14520). 212 of 212 (NT; non-tumor) and 222 of 222 (Tumor) patients data were used for analysis. $P < 0.0001$ *vs*. adjacent normal tissue. **(B)** Overall survival (months) analyzed by Kaplan–Meier curve in HCC-bearing patients (GSE54236) expressing low and high *SHBG* transcription levels. Scatter plots of tumor SHBG transcription levels (high and low) classified by average. NT and tumor SHBG transcription levels are indicated for comparison. Each average is indicated as a yellow line. 32 of 32 (low SHBG) and 49 of 49 (high SHBG) patients data were used for analysis. $P = 0.002$ *vs*. High SHBG HCC-bearing patients. **(C)** Representative gross and histological images showing HCC in diethylnitrosamine (DEN)-treated WT and *SHBG*-Tg mice. Hematoxylin and eosin (H&E) staining (Scale bar, 400 μm) is shown. *SHBG* expression and protein are presented. **(D)** Tumor numbers of DEN-treated WT and *SHBG*-Tg mice. **(E)** Tumor burden per liver (Sum) of DEN-treated WT and *SHBG*-Tg mice. Areas were quantified by Image J. **(F)** Western blot analysis and quantification of HMGB1 in plasma of DEN-treated WT and *SHBG*-Tg mice. Plasma albumin was used for an internal control. **(G)** Western blot analysis and quantification of intact and cleaved form of PARP, and PCNA in tumor of DEN-treated WT and *SHBG*-Tg mice. GAPDH was used for an internal control. **(H)** Immunostaining of TUNEL (green) as a marker of apoptosis in tumor of DEN-treated WT and *SHBG*-Tg mice (Scale bar, 50 μm). Nuclei are stained with DAPI (blue). **(I)** mRNA expression of apoptotic genes in tumor of DEN-treated WT and *SHBG*-Tg mice. *Rplp0* was used for an internal control. **(J)** Immunostaining of Ki67 (pink) as a marker of proliferation in tumor of DEN-treated WT and *SHBG*-Tg mice (Scale bar, 50 μm). Nuclei are stained with DAPI (blue). **(K)** mRNA expression of proliferation genes in tumor of DEN-treated WT and *SHBG*-Tg mice. *Rplp0* was used for an internal control. **(L)** Plasma IL-6 and IL-1β levels in DEN-treated WT and *SHBG*-Tg mice. **(M)** IL-6 and IL-1β levels in supernatant of Raw 264.7 cells. 1% charcoal-dextran (CD)-treated plasma and necrotic cell debris were treated for 3 h after steroid starvation. **(N)** IL-6 and IL-1β levels in supernatant of Raw 264.7 cells. Pure SHBG and necrotic cell debris were treated for 3 h after steroid starvation. **(O)** Plasma testosterone level in DEN-treated WT and *SHBG*-Tg mice. **(P)** Western blot analysis and quantification of AR in tumor of DEN-treated WT and *SHBG*-Tg mice. β-actin was used for an internal control. **(Q)** mRNA expression of proliferation genes in pRC/CMV or *SHBG* vector-transfected SNU423 cells. *Rplp0* was used for an internal control. **(R)** Cell proliferation rate of pRC/CMV or *SHBG* vector-transfected SNU423 cells after steroid starvation. **(S)** Western blot analysis and quantification of intact and cleaved forms of PARP in pRC/CMV or *SHBG* vector-transfected SNU423 cells. β-actin was used for an internal control. **(T)** Western blot analysis of MERTK, pMERTK, ERK1/2, JNK, p38, pERK1/2, pJNK, pp38, C-JUN, and C-FOS. β-actin was used for an internal control. Student's *t*-test and one-way ANOVA followed by a tukey's multiple comparison test were used for analysis. Values represent means ± SD. * $P < 0.05$. Data were quantified from replicated values in which independent experiments were performed in triplicate at least.

overexpression reduced nuclear AR and AR target gene levels in SNU423 cells (Fig. S8C, D). Accordingly, plasma SHBG is shown to suppress androgenic action following the free-hormone hypothesis. This could influence the cancer cell survival because testosterone increases HCC cell growth in AR overexpressed SNU423 cells (Fig. S9). Indeed, *SHBG*-overexpressed cells significantly suppressed the cell growth in testosterone treatments (Fig. S8E) and increased apoptotic PARP regardless of androgen regulation (Fig. S8F, G).

Hence, the following study focused on that SHBG had a steroid-independent role in the suppression of cancer cell proliferation. *SHBG*-overexpressed SNU423 cells decreased proliferation-related transcripts and cell proliferation rates without steroid hormones (Fig. 1Q, R). In DAMPs incubation, *SHBG*-overexpressed cells increased apoptotic PARP and suppressed proliferation rates (Fig. 1S, S10). These results imply that SHBG independently restrains cell proliferation in HCC beyond the canonical suppressive role of testosterone modulator. Substantially, *SHBG*-tumors, *SHBG*-overexpressed SNU423 cells, and SHBG-treated Raw 264.7 cells suppressed MERTK phosphorylation, which relates to suppression of cancer cell death and promotion of cancer cell proliferation. These might be related to the extracellular accumulation of SHBG, which can hinder the DAMPs-mediated MERTK activation since the SHBG domain of Gas 6 binds to MERTK.[3] MERTK phosphorylates mitogen-activated protein kinases (MAPK) which are responsible for *C-Jun* and *C-Fos* expression and the nucleus translocation of AP-1, promoting the proliferation of cancer cells.[4] Under the SHBG condition, MAPKs activation and AP-1 levels were substantially decreased following low MERTK phosphorylation, suggesting that SHBG is a crucial regulator for HCC progression *via* the MERTK-AP-1 axis (Fig. 1T, S11,12).

Collectively, the present study reveals the SHBG's role during HCC progression in the context of steroid-dependent and steroid-independent regulation. As a crucial modulator for androgens, SHBG suppresses androgen-mediated cancer cell proliferation in AR dominance. Beyond its role as a steroid regulatory protein, SHBG independently increases apoptosis and decreases proliferation of cancer cells, and suppresses pro-inflammatory IL-1β production of macrophage. MERTK phosphorylation and JNK/p38/ERK signals were substantially suppressed by SHBG, thereby lowering activation of AP-1 complex which is closely associated with cancer cell proliferation and IL-1β production of immune cells.[5] Through our findings of SHBG physiology, as a clinical marker and therapeutic strategy for HCC, SHBG should be highlighted for suppressing cancer cell proliferation and pro-inflammatory response in the HCC environment.

Author contributions

SRL and EJH designed research; SRL and EJH performed research; SRL and EJH analyzed data; SRL and EJH wrote the paper.

Conflict of interests

Authors declare no conflict of interests.

Funding

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (No. 2018R1D1A1A02043102 and 2021R1I1A2042991). This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIT) (No. 2021R1A4A1033078). This work was supported by the NRF (National Research Foundation of Korea) Grant funded by the Korean Government (NRF-2019-Global Ph.D. Fellowship Program).

Acknowledgements

We thank Dr. Geoffrey L. Hammond for providing SHBG mice and mentoring SHBG physiology.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2021.10.003.

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30 August 2021
Available online 1 November 2021