SerpinB5 interacts with KHDRBS3 and FBXO32 in gastric cancer cells

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Abstract. Mammary serine protease inhibitor B5 (SerpinB5) is a potential oncogene in gastric cancer (GC); however, the molecular mechanism by which SerpinB5 promotes oncogenesis remains elusive. In this study, SerpinB5-associated proteins were selected based on yeast two-hybrid screening and microarray analysis after RNA interference and were validated using co-immunoprecipitation (Co-IP) and RNA Co-IP. The expression profiles of the interacting proteins were analyzed by Western blotting and immunohistochemistry. The effects of SerpinB5 on KHDRBS3 and FBXO32 expression in GC cells were analyzed using real-time PCR and Western blotting after the expression of SerpinB5 was modified. By yeast two-hybrid screening and microarray analysis, FBXO32 and KHDRBS3 were found to be SerpinB5-interacting proteins. The interactions were confirmed by Co-IP. An RNA co-immunoprecipitation assay found that KHDRBS3 interacted with *FBXO32* mRNA. The expression of SerpinB5 was much stronger in the nucleus of GC cells. FBXO32 was expressed at higher levels in the cytoplasm of GC cells. KHDRBS3 was primarily detected in the nucleus of normal mucosal cells. *SerpinB5* expression was modified in GC cells, *KHDRBS3* mRNA levels remained stable, however, *FBXO32* mRNA levels changed 24 h after changes in KHDRBS3 protein levels were detected. In conclusion, SerpinB5 interacts with KHDRBS3 and FBXO32, and KHDRBS3 can interact with *FBXO32* mRNA.

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Introduction

Mammary serine protease inhibitor B5 (SerpinB5) is a 42-kDa protein that is a member of the ovalbumin clade of serine protease inhibitors (serpins). SerpinB5 was identified by subtractive hybridization and differential display, and was found to be expressed in normal mammary epithelial cells but not in most mammary carcinoma cell lines, and was considered to be a tumor suppressor (1).

However, conflicting reports on its function in cancer occurrence and progression have been reported. Gastric tumor specimens showed increasing SerpinB5 expression level compared to corresponding normal tissues. The frequency of SerpinB5 induction was associated with the stage of gastric cancer (GC) and lymph node metastasis. SerpinB5 may have an important role in the progression and metastasis of gastric adenocarcinoma (2,3).

The function of SerpinB5 and its significance have not been fully elucidated in human GC. The focus of this study was to identify SerpinB5-associated molecules. Using RNA interference techniques, microarray and yeast two-hybrid screening, we found that KHDRBS3 and FBXO32 specifically interacted with SerpinB5. These results not only suggest a possible mechanism for SerpinB5 in GC but also provide new avenues for SerpinB5-based drug development.

Materials and methods

Chemicals and reagents. Antibodies were purchased from commercial sources and included SerpinB5 monoclonal antibody (Novocastra, UK), TTK (N1) monoclonal antibody (Santa Cruz Biotechnology, USA), FBXO32 polyclonal antibody (Santa Cruz Biotechnology), KHDRBS3 polyclonal antibody (Santa Cruz Biotechnology), DDX18 polyclonal antibody (Abnova, Taiwan, China), and GAPDH monoclonal antibody (Abcam, USA). The kits used for analysis were an EZ-10 Spin Column Plasmid DNA Miniprep kit (Bio Basic Inc., Canada), a Plasmid Maxi Preparation kit (Qiagen, USA), Matchmaker™ Library Construction & Screening kits (Clontech Laboratories, USA), a Matchmaker AD LD-Insert Screening Amplimer Set

(Clontech Laboratories), an Advantage® 2 PCR kit (Clontech Laboratories), and a Mammalian Co-Immunoprecipitation kit (Pierce, USA). Other reagents used were Lipofectamine 2000 (Invitrogen, USA), dimethyl sulfoxide (DMSO) (Sigma, USA), X-α-Gal (Clontech Laboratories), Minimal SD Base without agar (Clontech Laboratories), 3-amino-1,2,4-triazole (3-AT) (Sigma), Adenine hemisulfate (Sigma), -Trp DO Supplement (Clontech Laboratories), -His/-Leu DO Supplement (Clontech Laboratories), and -His/-Leu/-Trp DO Supplement (Clontech Laboratories).

Human tissues and cell cultures. Extracts or paraffin-embedded samples of histologically confirmed human GC tumor tissues and the matching normal tissues were obtained with informed consent from five patients who underwent radical resection of GC in July 2006 at the Department of Surgery, Ruijin Hospital, Shanghai, China. The human gastric mucosa cell line GES-1 (from Cell Bank of Chinese Academy of Sciences) and the GC cell lines KATOIII and SUN-16 (from American Type Culture Collection) were cultured at 37˚C with 5% CO_2 in RPMI-1640 (Giboco BRL, USA) containing 10% fetal bovine serum (Sigma).

Real-time PCR. Real-time PCR reactions were performed according to a previously reported protocol (4). The primer sequences were: sense 5'-GTTCCAGACATTCTCGCTTC-3' and anti-sense 5'-ATAGTAGCCTGAGCATGTGC-3' for *SerpinB5* (107 bp); sense 5'-GGACCTGACCTGCCGTCTAG-3' and anti-sense 5'-GTAGCCCAGGATGCCCTTGA-3' for *GAPDH* (100 bp).

The results of the real-time PCR data are represented as Ct values. The relative changes in gene expression were calculated by the ΔΔCt method (5).

RT-PCR. RT-PCR was used to prepare full-length *SerpinB5* cDNA for yeast two-hybrid screening and for the detection of *FBXO32* in RNA by co-immunoprecipitation, which was performed as previously reported (4). The primer sequences were: sense 5'-ccggCATATGATGGATGCCCTGCAACTAGC-3' and anti-sense 5'-gctgGTCGACCTATGCCACTTAAGGAGAAC-3' for *SerpinB5*; sense 5'-GAAGCGCTTCCTGGATGAGA-3' and anti-sense 5'-GGAATCCAGAATGGCAGTTG-3' for *FBXO32*; sense 5'-TGGGCATGGGTCAGAAGGA-3' and anti-sense 5'-AAGCATTTGCGGTGGACGATGGAGG-3' for β-actin. The RT-PCR products were resolved by electrophoresis on a 1.5% agarose gel and were stained with ethidium bromide.

Western blotting. Western blotting was performed according to our previously reported protocol (4).

Immunohistochemistry. Immunohistochemistry (IHC) was performed according to our previously reported protocol (6).

RNA interference. Silencing of SerpinB5 was achieved by transfection with siRNA duplexes, targeting SerpinB5. SerpinB5-specific siRNAs included siRNA1 (5'-ACAGUAA CAUCGGAUGUAAtt-3'), siRNA2 (5'-GGAAUCACGU UAGAGGAAAtt-3') and siRNA3 (5'-CUUGUCUCUUCAU CUAAUAtt-3'). A non-silencing oligonucleotide (5'-UUCUC CGAACGUGUCACGUtt-3') was used as a specificity siRNA control. All siRNAs were chemically synthesized by Shanghai GeneChem Co. Ltd. GC cells in 12-well cell dish were transfected with siRNA complexed to Lipofectamine 2000.

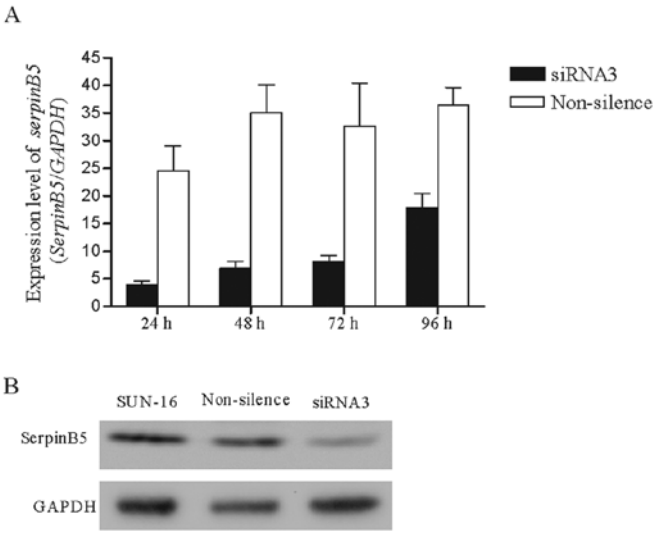

Figure 1. Downregulation of SerpinB5 expression using siRNA oligonucleotides. (A) The maximum suppressive effect using the siRNA3 was ~84.1% in SUN-16 cells. (B) SerpinB5 was significantly downregulated at the protein level by the siRNA3 48-h post transfection.

Microarrays analysis. Microarrays were used to analyze the changes in the mRNA expression profile after SerpinB5 was downregulated in KATOIII and SUN-16 GC cells. The cDNA microarray used in the present study consisted of 12,630 cDNA clones representing 10,647 genes. This microarray was the same as the one used by Zheng *et al* (7). The microarray and the experimental procedures have been confirmed to be feasible by previous studies (7,8). The hybridization and scan procedures were the same as described previously (8). A conservative, two-fold change threshold (i.e., SerpinB5-specific siRNA treated GC cell samples versus untreated GC cell samples) was used to determine regulated genes.

Yeast two-hybrid screening. Yeast two-hybrid screening was performed using Matchmaker™ Library Construction & Screening kits, following the manufacture's protocol. The matching of normal gastric tissue from a 52-year-old GC patient was used to prepare full-length *SerpinB5* cDNA by RT-PCR after RNA extraction. The bait vector was created by cloning the full-length *SerpinB5* cDNA into the Gal4 DNA-binding domain vector pGBKT7 to yield pGBKT7-SerpinB5. The GC tissue from a 68-year-old GC patient was used to generate a cDNA library for yeast two-hybrid screening. The positive interactions were analyzed by PCR colony screening using the Matchmaker AD LD-Insert Screening Amplimer Set and the Advantage 2 PCR Polymerase Mix, according to the manufacturer's protocol. The cDNA insert was analyzed by agarose/EtBr gel electrophoresis and sequencing.

Co-immunoprecipitation. Co-Immunoprecipitation (Co-IP) experiments were performed using the Pierce Mammalian Co-immunoprecipitation kit according to the manufacturer's

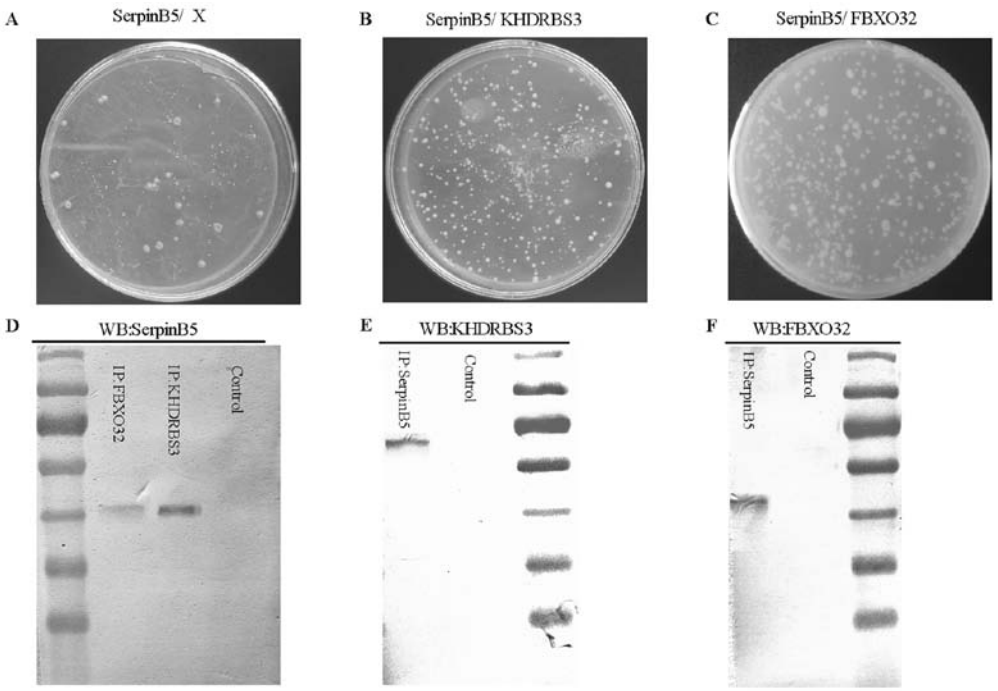

Figure 2. Identification of KHDRBS3 and FBXO32 as interactors of SerpinB5. A cDNA library derived from GC tissue was screened by the co-transformation method using Matchmaker Library Construction & Screening kits from Clontech Laboratories (A). The interactions between KHDRBS3, FBXO32 and SerpinB5 were retested in yeast by the co-transformation method (B and C). KHDRBS3, FBXO32 (D) and SerpinB5 (E and F) were used as bait protein, and we confirmed that KHDRBS3 and FBXO32 could interact with SerpinB5 by co-immunoprecipitation.

protocol. Briefly, bait protein antibody was immobilized overnight to the antibody-coupling gel according to the manufacturer's instructions (100 μg or 100 μl antibody per 100 μl coupling gel). The bait and prey protein complex was subsequently precipitated from mammalian cell lysis buffer using an immobilized bait protein antibody. It was analyzed by Western blotting after elution.

RNA co-immunoprecipitation. Untreated SUN-16 cells and GC tissues were lysed in 500-μl lysis buffer [50 mM Tris-HCl (pH 7.5), 2 mM EDTA, 120 mM NaCl, 10% glycerol, and 0.5% NP-40]. Whole lysate was mixed with 50 μl protein A sepharose beads (Rockland Immunochemicals, USA) and pre-bound with KHDRBS3 antibody for 5 h at 4˚C. The beads were washed and split with buffer B70 (9). Half of the bead solution was prepared for Western blot analysis. The remaining half was extracted with TRIzol reagent to isolate RNA. Then, FBXO32 was detected by RT-PCR with FBXO32-specific primers.

Statistical analysis for real-time PCR. The results of the suppressive effect of siRNA were evaluated by one-way ANOVA. Statistical analyses for real-time PCR were performed with software from SPSS 10.0 for Windows (Chicago, IL, USA). $p<0.05$ was considered significant.

Results

Identification of KHDRBS3 and FBXO32 as target genes for SerpinB5. The SerpinB5 mRNA level in GC cells was modified by RNA interference (RNAi) with 20 nM of siRNA1, siRNA2 and siRNA3. Of three siRNAs, siRNA3 was validated to be the most efficient. The *SerpinB5* expression level in siRNA3-treated SUN-16 GC cells was reduced ~84.1% from the non-silenced SUN-16 cells (Fig. 1A). The data demonstrate that the protein expression of SerpinB5 was significantly downregulated by the siRNA3 (Fig. 1B).

Table I. List of differentially expressed genes analyzed by microarray analysis and yeast two-hybrid screening.

Gene	Fold-change by microarray
DDX18	(+) 2.1451
FBXO32	(-) 3.0694
KHDRBS3	(+) 3.0630
TTK	(+) 3.2339

(+), upregulated; (-), downregulated.

Total mRNA was extracted from KATOIII and SUN-16 GC cells that were transfected with a 20-nM siRNA3 and were harvested 60 h after transfection; it was then used in mRNA expression profiling by microarray. mRNA from untreated KATOIII and SUN-16 GC cells was used as the reference control in the microarray experiments. There were 210 upregulated genes and 108 downregulated genes in the siRNA-treated GC cells.

To identify the molecular targets of SerpinB5, we screened a cDNA library derived from a GC sample using a yeast two-hybrid screen (Fig. 2A). Fifty-three genes were identified as candidate SerpinB5-interacting proteins, based on a positive two-hybrid interaction kit, as determined by PCR colony-screening and

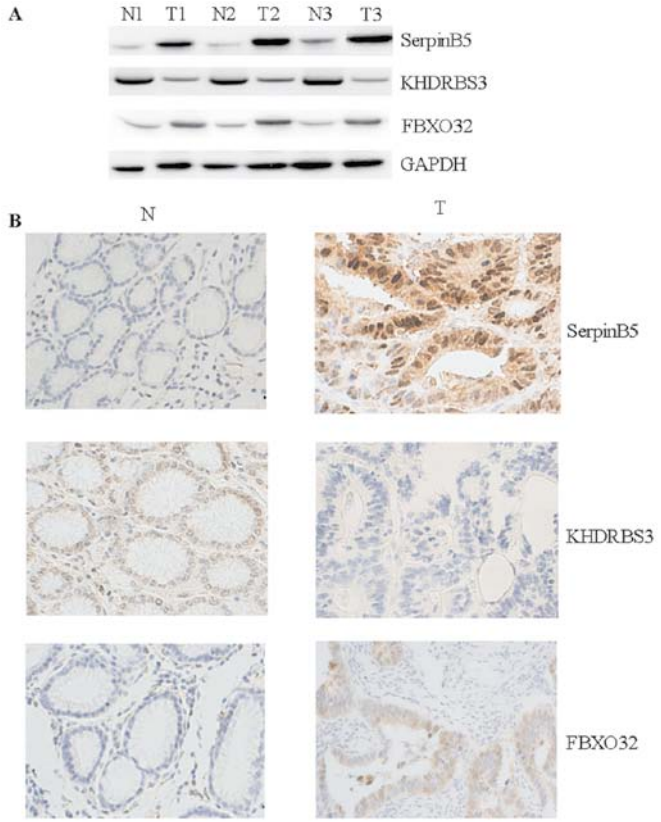

Figure 3. SerpinB5, KHDRBS3 and FBXO32 expression profiles in GC tissue and normal gastric tissue. Proteins were extracted from matching normal (N) and tumor (T) gastric tissues of three patients (N 1-3, T 1-3). Western blot analysis of SerpinB5, KHDRBS3 and FBXO32 (A) showed higher expression of SerpinB5 and FBXO32 in GC tissue than in normal gastric tissue. The expression of KHDRBS3 was the reverse to that seen with SerpinB5. GAPDH was used as a protein loading control. These results were confirmed by IHC analysis (B). IHC analysis showed that SerpinB5 and FBXO32 were expressed in both the nucleus and cytoplasm of GC cells. SerpinB5 expression was stronger in the nucleus and FBXO32 expression was stronger in the cytoplasm. KHDRBS3 was expressed primarily in the nucleus of normal gastric tissue.

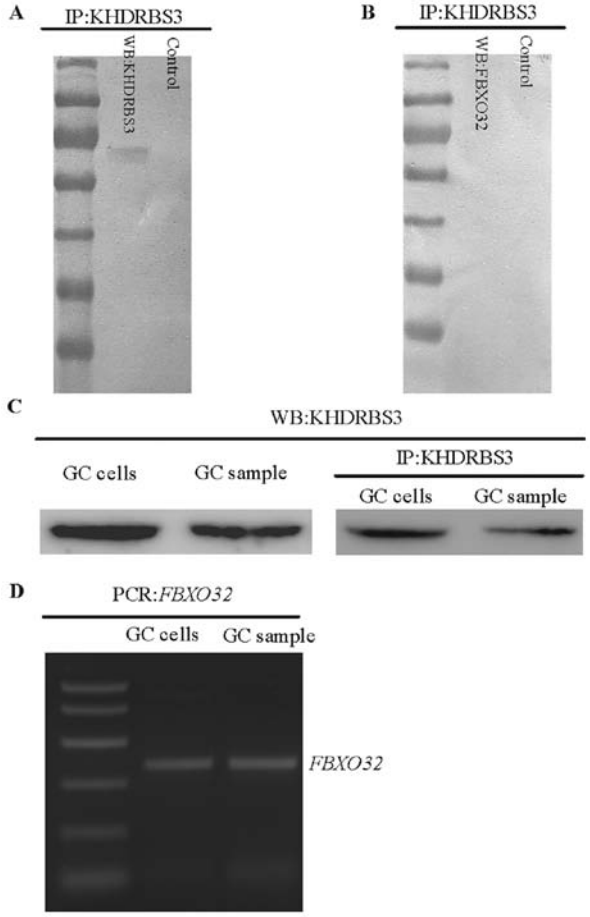

Figure 4. KHDRBS3 interacts with *FBXO32* in GC cells. Co-immunoprecipitation was used to test for the interaction of KHDRBS3 and FBXO32; however, no positive interaction was detected (A and B). KHDRBS3 was immunoprecipitated with antibody from SUN-16 cells and the GC sample, and identified by Western blotting (C). RNAs that co-immunoprecipitated with KHDRBS3 were extracted. *FBXO32* was detected by RT-PCR with *FBXO32*-specific primers (D).

DNA sequencing analysis. However, there were only four genes (i.e., *DDX18, FBXO32, KHDRBS3*, and *TTK*) in the differentially expressed gene list that were obtained from the microarray experiments (Table I).

The interactions between KHDRBS3 (Fig. 2B), FBXO32 (Fig. 2C) and SerpinB5 were retested in yeast by the co-transformation method according to the manufacturer's protocol. Using co-immunoprecipitation assays, we found that KHDRBS3 and FBXO32 interacted with SerpinB5 (Fig. 2D-F).

SerpinB5, KHDRBS3 and FBXO32 expression in GC. SerpinB5, KHDRBS3 and FBXO32 were detectable in all tissues. SerpinB5 was expressed at higher levels in GC samples than corresponding normal tissues. FBXO32 showed the same expressional profile as SerpinB5, while KHDRBS3 had the inverse profile (Fig. 3A). These data were confirmed by immunohistochemistry. SerpinB5 was expressed in both the nucleus and cytoplasm of GC cells; however, the expression level was much stronger in the nucleus. FBXO32 was expressed at higher levels in the cytoplasm. KHDRBS3 was primarily detected in the nucleus of normal mucosal cells (Fig. 3B).

KHDRBS3 interacts with FBXO32 mRNA rather than the FBXO32 protein. Co-IP experiments found that KHDRBS3 did not interact with FBXO32 (Fig. 4A and B). Western blotting and whole cell lysates (WCL) identified KHDRBS3 at ~55 kDa in SUN-16 cells and the GC sample (Fig. 4C left). KHDRBS3 was immunoprecipitated with the antibody from SUN-16 cells and GC sample (Fig. 4C right). RNAs that co-immunoprecipitated with KHDRBS3 were extracted. *FBXO32* could be detected by RT-PCR with *FBXO32*-specific primers (Fig. 4D).

Effects of SerpinB5 on KHDRBS3 and FBXO32 expression in GC cells. SerpinB5 showed a higher expression in SUN-16 than GES-1 at both the mRNA and protein levels (Fig. 5A and B). The RNA expression level of *KHDRBS3* did not show a significant difference after transfection with the siRNA or with pGBKT7-SerpinB5, while *FBXO32* levels changed along with *SerpinB5* levels 72 h after transfection (Fig. 5C and D). SerpinB5 expression was knocked down by the siRNAs in SUN-16 cells. While KHDRBS3 protein level increased from 48 to 120 h after transfection with the siRNAs. The expression level of FBXO32 decreased drastically after 72 h of transfection with the SerpinB5-specific siRNA3. (Fig. 5E). SerpinB5

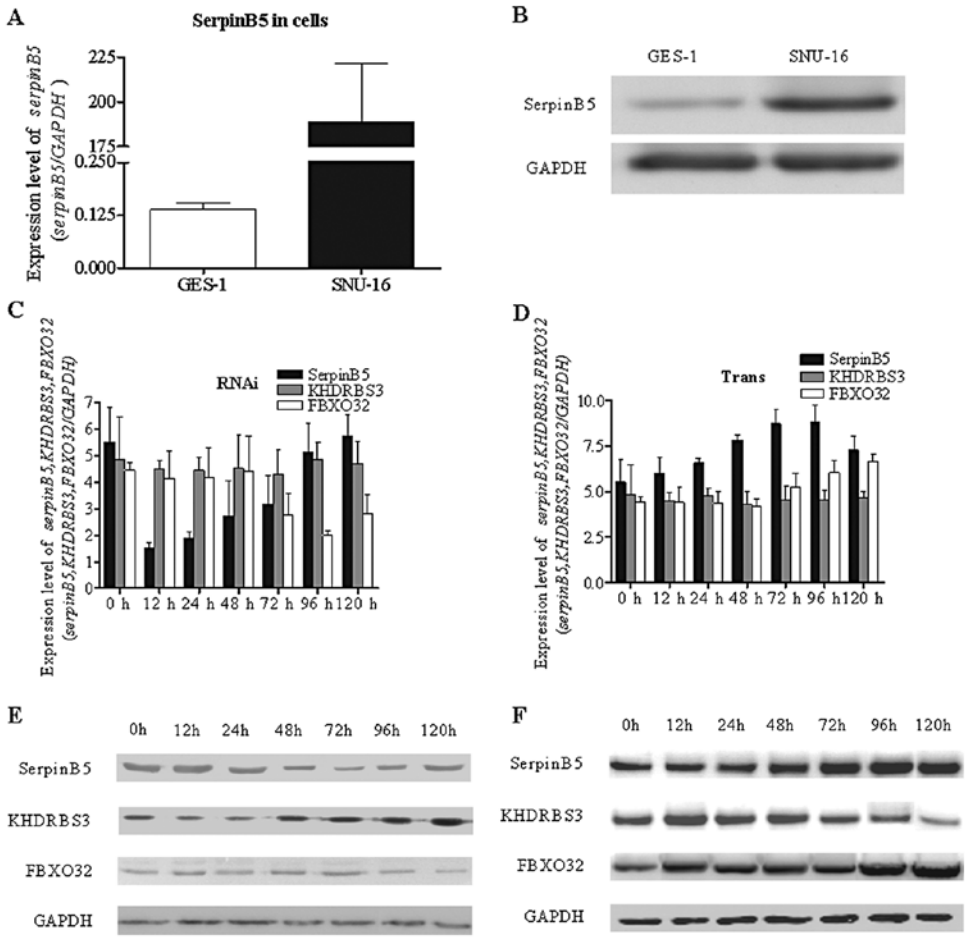

Figure 5. Real-time PCR and Western blot analysis showing changes in KHDRBS3 and FBXO32 expression following the SerpinB5-specific siRNA and pGBKT7-SerpinB5 transfection SUN-16 cells. SerpinB5 expression was much stronger in SUN-16 than in GES-1, both at the mRNA and protein levels (A and B). The RNA expression level of *KHDRBS3* did not show a significant difference after *SerpinB5* being regulated, while *FBXO32* levels changed along with *SerpinB5* levels 72 h after transfection (C and D). KHDRBS3 protein level increased from 48 to 120 h after transfection with the siRNAs. The expression level of FBXO32 decreased drastically after 72 h of transfection with the SerpinB5-specific siRNA3 (E). KHDRBS3 protein levels decreased from 48 to 120 h after pGBKT7-SerpinB5 transfection. FBXO32 protein levels increased drastically after 72 h of pGBKT-SerpinB5 transfection (F).

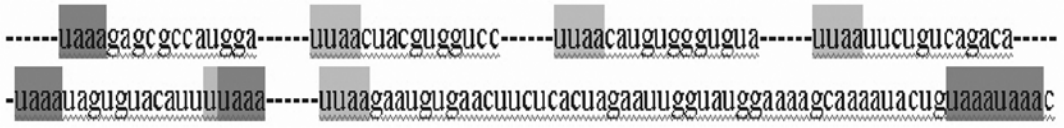

Figure 6. The U(U/A)AA motifs in *FBXO32*.

was overexpressed by transiently transfecting the pGBKT7-SerpinB5 vector with Lipofectamine 2000. KHDRBS3 protein levels decreased from 48 to 120 h after pGBKT7-SerpinB5 transfection. FBXO32 protein levels increased drastically after 72 h of pGBKT-SerpinB5 transfection (Fig. 5F).

Discussion

The paradoxical expression profile of SerpinB5 in GC suggests that it has functions beyond its ability to act as a tumor suppressor, and its mechanism of action in GC is important for comprehending the panoramic function of SerpinB5 in tumor occurrence and progression. The aim of this study was to identify the SerpinB5-associated proteins in GC. We screened for candidate SerpinB5-interacting proteins by microarray analysis of GC cells following SerpinB5 knockdown by RNA interference and by the yeast two-hybrid assay, using a cDNA library derived from a GC sample. We identified DDX18, FBXO32, KHDRBS3 and TTK as novel interacting proteins. The yeast two-hybrid system is a powerful and sensitive molecular genetics approach for studying protein-protein interactions that was developed for high-throughput screening during the early 1990s (10,11). Usually high throughput two-hybrid data are notorious for detecting false positives (12). Co-IP, in which proteins of interest are co-purified with the protein of study, is a good way to address this important issue (13). Only the interactions between KHDRBS3, FBXO32 and SerpinB5 were verified by Co-IP. KHDRBS3 interacted with *FBXO32* mRNA as determined by RNA Co-IP and RT-PCR analysis. After *SerpinB5* expression was modified in GC cells, *KHDRBS3*

mRNA levels remained stable, however, *FBXO32* mRNA levels changed 24 h after changes in KHDRBS3 protein levels could be detected. This might indicate that KHDRBS3 affects *FBXO32* mRNA (Fig. 5).

KHDRBS3 maps to 8q24.2 and shares the same basic structure as Sam68 (14). Similar to Sam 68, KHDRBS3 possesses RNA binding activity. The predominantly nuclear localization of KHDRBS3 (Fig. 3) suggests that KHDRBS3 may shuttle between the cytoplasm and the nucleus, as has been shown for other predominantly localized RNA-binding proteins (14,15). A high-affinity RNA motif, *U(U/A)AA*, has been identified for KHDRBS3 using SELEX (16,17). We examined the sequence of FBXO32 and found that it is abundant in *U(U/A)AA* motifs (Fig. 6).

The biological functions of SerpinB5 as a tumor suppressor have been previously reviewed (18). SerpinB5 acts as a tumor suppressor through its binding partners, which include tissue-type plasminogen activator (19), types I and III collagen (20), interferon regulatory factor 6 (IRF6) (21), glutathione s-transferase (GST) (22), pro-uPA (23), histone deacetylase 1 (HDAC1) (24), nuclear IKKalpha (25), PTEN, p53 (26), and testisin (27). However, SerpinB5 is believed to be an oncogene in GC, and the expression profiles of SerpinB5 in different types of GC have been extensively examined (2,3,28,29). Our data show that SerpinB5 might act as an oncogene though its interaction with KHDRBS3 and FBXO32.

In conclusion, we show for the first time that SerpinB5 interacts with KHDRBS3 and FBXO32 and that KHDRBS3 could interact with *FBXO32* mRNA. These novel findings may point to an exciting new direction for future mechanistic studies and SerpinB5-based drug development in GC.

Acknowledgements

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