Abstract. – OBJECTIVE: Thrombospondin 2 (THBS2) expression and its prognostic value have been documented in several types of cancer. Nevertheless, the potential role and clinical significance of THBS2 in uveal melanoma (UM) have never been reported. Thus, in our study, we aimed to explore the clinical significance and prognostic impact of THBS2 in UM.

MATERIALS AND METHODS: Survival and prognosis analyses were implemented using the Kaplan-Meier method and COX’s proportional hazards model based on the clinical data retrieved from The Cancer Genome Atlas (TCGA) database. Colony formation, cell proliferation, invasion and migration assays in M23 cell line were performed to evaluate the effects of THBS2 on UM in vitro. To further reveal whether the dysregulated THBS2 expression regulates the UM metastasis, protein biomarkers including serine-threonine kinase (AKT), p-AKT, phosphoinositide 3-kinase (PI3K), p-PI3K, and p70S6K were measured using Western blotting analysis.

RESULTS: THBS2 was up-regulated in metastatic UMs. Relationship of THBS2 expression level with the clinicopathological factors demonstrated that the expression level of THBS2 was significantly correlated to histological type, recurrence, and dead. Univariate as well as multivariate COX analyses demonstrated that THBS2 could serve as an independent prognostic factor for overall survival of UM. The knockdown of THBS2 significantly inhibited the proliferation rate of M23 cells, suppressed the colony numbers of M23 cells, lowered the invasive and migratory cell proportion. Importantly, Western blotting results implicated that THBS2 knockdown significantly decreased the expression level of p-AKT, p-PI3K, and p70S6K in M23 cell line.

CONCLUSIONS: This is the first study to report that THBS2 may play important roles in UM progression and might be a novel prognosis biomarker for UM.

Key Words: Uveal melanoma, Diagnosis, Prognosis, THBS2, PI3K signaling pathway.

Introduction

Uveal melanoma (UM), as the most common primary malignant eye tumor, represents about 5% of all melanomas. Currently, various treatments are available for managing UM, including radioactive plaque therapy, and thermotherapy. However, the prognosis for UM patients is unfavorable, particularly in the metastatic phase. So far, the most common metastasis site is the hepatic, reported in 87% of metastasis cases. Approximately 40-50% of UM patients deaths are the results of metastasis, even with early diagnosis as well as proper therapy. The biology of UM onset and progression is unknown. Therefore, it is an urgent necessity to explore the molecular metastatic events of UM development and to seek for the novel therapeutic target of UM.

Thrombospondins (THBSs) play important roles in a wide range of settings which involve in angiogenesis, cell-extracellular matrix interactions, as well as tumor formation. THBS2, one member of THBSs, has been reported to mediate the antiangiogenic activity and to inhibit the formation of focal adhesions in endothelial cells. Moreover, THBS2 has been suggested to interact with the endothelial cell membrane protein CD36 to further suppress migration and induce apoptosis. The THBS2 expression and its prognostic value have been reported in several types of cancer, for example, colon cancer, lung cancer, pros-
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tate cancer, and gastric cancer\textsuperscript{10-13}. Significantly, THBS2 mRNA expression has been demonstrated to be associated with the tumor-node-metastasis in gastric cancer\textsuperscript{14}. In a colorectal cancer study, the THBS2 expression has been reported to be negatively correlated with liver metastasis\textsuperscript{15}. These studies investigating THBS2 expression in the prognosis in various tumors yielded inconsistent results. Moreover, to the best of our knowledge, no report has measured the expression and prognosis of THBS2 in UMs. Hence, in our study, to examine the THBS2 expression level in human UMs and then to explore the possible correlations between the THBS2 expression and clinicopathological features, we conducted the data analysis based on Gene Expression Omnibus (GEO) and The Cancer Genome Atlas (TCGA) databases. In an attempt to verify our findings, colony formation assay, proliferation experiment, cell invasion, and migration assay were performed in M23 cell line to explore the effect of THBS2 in UM in vitro. The Western blotting analysis was used to examine the expression level of the serine-threonine kinase (AKT), p-AKT, phosphoinositide 3-kinase (PI3K), p-PI3K, and p70S6K to reveal whether the dysregulated THBS2 expression regulates the UM metastasis. Our results suggest that the expression of THBS2 might be closely related to tumor metastasis and prognosis.

Materials and Methods

Data Mining of Transcriptomic Data from the GEO Database

A transcriptomic data (GSE39717), comprising 39 primary UM cases and 2 metastatic melanoma cases from the GEO database, was selected for research. In our study, the THBS2 expression level in these two groups was generated.

Data Mining in the TCGA

The data of patients with primary UM in TCGA were downloaded, covering the clinical information of 80 UM patients. The data were downloaded on 5\textsuperscript{th} Oct 2017.

Cell Culture

The human UM cell lines SP6, M23, OCM-1A, and C918 and normal melanocyte cell line (D78) cells were obtained from the Shanghai Cell Bank at the Chinese Academy of Sciences (Shanghai, China). These cells were incubated in Roswell Park Memorial Institute (RPMI)-1640 media supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 0.1 mg/ml streptomycin at 37°C in a 5% CO\textsubscript{2} incubator.

Knockdown of THBS2 in UM Cell Line and Transfection

The up-regulated THBS2 expression UM cell line M23 was used to establish the stable THBS2 knockdown cell line. Small interfering RNA (siRNA) targeting THBS2 (Si-THBS2) and a negative control siRNA were designed and generated by Shanghai GenePharma (Shanghai, China). According to the manufacturer’s protocol, transfection of siRNA (sense (5'-GUUUGCUCAGAAGGATT-3') and antisense (5'-UCCGAACGUGACATT-3')) was performed by means of Lipofectamine 2000 (Invitrogen). Interference efficiency was determined by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) and Western blotting.

RNA Extraction and qRT-PCR

Total RNA of cell line M23 was extracted using TRIzol reagent (Invitrogen, Carlsbad, USA). The primers sequences were: THBS2, forward: 5'-ATTCGCCATCAAGGCAAGGA-3', reverse: 5'-CAAAACCTACAGCGATGCCG-3'; GAPDH, forward: 5'-GGAGCGAGATCCCTCCAAAAT-3', reverse: 5'-GGCTGTTGTCATACTTCTCATGG-3'. The PCR was conducted based on RCR detection system with the following conditions: 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 60°C for 45 s, and 72°C for 30 min to detect the THBS2 and Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) expression level. The \(2^{-\Delta\Delta Ct}\) method was utilized to quantify the transcripts and normalized to GAPDH.

Cell Viability and Colony Formation Assay

Cell proliferation ability was assessed on 24 h, 48 h, 72 h, and 96 h after THBS2 knockdown using the cell counting assay kit-8 (CCK-8) following the protocol of a previous report\textsuperscript{16}. Cell viability was evaluated at the time of 24, 48, 72, and 96 h, and the absorbance at 450 nm was recorded. Colony formation was conducted as described previously\textsuperscript{17,18}. In brief, 8 × 10\textsuperscript{3} si-THBS2 and si-control cells were incubated in the medium containing 10% FBS, and replacing the media every four days. The plates were cultured at 37°C in a 5% CO\textsubscript{2} incubator for 14 days. Then, the colo-
nies were fixed using 4% paraformaldehyde and stained using 0.1% crystal violet (Sigma-Aldrich, St. Louis, MO). Next, the colonies with more than 50 cells were counted. The experiments were done at least three times.

**Wound Healing Assay, Cell Invasion, and Migration Assays**

Cells were cultured in plates to form a monolayer before the assay. Following making a scratch using a 10-µl pipette tip, the cells were washed with PBS. The cells were allowed to move for 24 h. The wounds were taken using a microscope (Olympus, Tokyo, Japan) at 0, and 24 h following wounding. The widths of the wounded areas were measured at 0 h (W₀) and 24 h (W₂₄). The relative migration distance was calculated as (W₀−W₂₄)/W₀ x 100%. At least 5 random areas were recorded.

To further study the effects of THBS2 on cell invasion and migration, transwell assays were conducted using 6.5 mm transwell chambers with 8.0 µm pore-size polycarbonate membranes with or without Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). In the invasion assay, the upper chambers were coated with 100 µl of Matrigel. In both invasion and migration assays, cells were suspended in serum-free medium at a 1:6 dilution (BD Biosciences), and incubated at 37°C for 4 h. Subsequently, cells were loaded onto the top chamber of the transwell at a density of 5×10⁵ cells/ml (200 µl/chamber). The lower chambers were filled with 500 µl complete media containing 10% FBS. After overnight incubation at 37°C in a 5% CO₂ atmosphere, non-invasive cells were removed from the top chambers with the cotton swabs. The remaining cells that were attached to the underside of the membranes were fixed using 4% paraformaldehyde and stained with 0.1% crystal violet for 20 min. Next, cells were counted in five randomly selected fields of view using a microscope (Nikon ECLIPSE 80i system), and the average value was analyzed for every field. Each experiment was conducted at least three times.

**Western Blotting Analysis**

Total proteins were extracted from the M23 cells and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and proteins were transferred onto polyvinylidene difluoride (PVDF) membranes. Then, we used the following antibodies to block and incubate: anti-THBS2 at 1:1000 dilution, anti-p-AKT at 1:1000, anti-p38K (1:1000), anti-p-p38K (1:1000), anti-p70S6K (1:1000), and anti-GAPDH (1:5000, GAPDH was used as an internal reference). Then, a horseradish peroxidase (HRP)-conjugated anti-rabbit IgG as the secondary anti-body (Santa Cruz Biotechnology, Santa Cruz, CA, USA, at 1:5000 dilution) was added to the membranes to continue to incubate for 2 h. All bands were detected using enhanced chemiluminescence (ECL) Western blot kit. Ultimately, Bio-Rad Quantity One 1-D software was used to quantify the protein bands.

**Statistical Analysis**

Statistical analyses were conducted using SPSS22.0 software in our study. The data were presented as the mean ± standard deviation (SD), and a chi-square test was used to perform the comparison between two groups, and one-way analysis of variance (ANOVA) test followed by a Student-Newman-Keuls post hoc test was utilized to compare the parameters of multi-groups. The survival curve was plotted based on Kaplan-Meier and log-rank test. COX hazards model was used to analyze the prognostic factors. Each variable with statistical significance in the univariate analysis was put into multivariate analysis models to assess the independent prognostic values on UM. Differences were defined to be statistically significant when p was set as 0.05.

**Figure 1.** THBS2 expression level in metastasis UM was significantly over-expressed. THBS2 expression level of patients with primary UM (n= 39) and metastasis UM (n= 2) from the GEO database was analyzed.
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**Results**

**THBS2 Was Up-Regulated in UM Metastatic Tissue**

By analyzing the published transcriptomic data of UM (GSE39717) obtained from the GEO database, we found that THBS2 was the significantly up-regulated by comparison between primary tumors and metastatic ones. Figure 1 showed that THBS2 was significantly over-expressed in metastatic tissue, relative to that in primary UM \((p = 6.28E-04)\). This suggested that abnormal THBS2 expression might be associated with UM metastasis.

**Relationship of THBS2 Expression Level With the Clinicopathological Factors in UM Patients**

To explore the clinical relevance of THBS2 expression in UM patients, we implemented the correlation analysis between THBS2 expression and clinicopathological factors, including age, histological type, tumor basal diameter, pathologic stage, pathologic-tumor, pathologic-node, pathologic-metastasis, recurrence, and dead. Table I listed the relationship of THBS2 level with the clinicopathological factors in UM. Based on this table, we found that the expression level of the THBS2 was significantly correlated with histological type \((p = 0.008)\), recurrence \((p = 0.005)\), and dead \((p = 0.007)\).

**Survival and Prognostic Impact Analyses for UM Patients**

The survival analysis is summarized in Figure 2. We observed that UM patients with high THBS2 expression had a worse prognosis, compared with those with a low level of THBS2 \((p = 0.000)\). Then, we conducted univariate and multivariate Cox proportional hazards analyses to prove the prognostic role of THBS2 for survival in UM patients (Table II). In univariate analysis, THBS2 expression \((p = 0.001)\), histological type \((p = 0.001)\), pathologic-metastasis \((p = 0.000)\), and recurrence \((p = 0.001)\) were significantly associated with shorter overall survival in UM patients. In multivariate analysis of the UM patients, THBS2 expression \((p = 0.017)\), and pathologic-metastasis \((p = 0.000)\) were predictive of overall survival. Multivariate analysis for UM demonstrated that THBS2 expression and pathologic-metastasis were the independent prognostic factors for UM.

**Figure 2.** Kaplan-Meier overall survival curves based on THBS2 expression level.

Table I. Clinical association between THBS2 expression and clinicopathological variables in uveal melanoma (UM) patients.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Low</th>
<th>High</th>
<th>(p)-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;60</td>
<td>19</td>
<td>16</td>
<td>0.435</td>
</tr>
<tr>
<td>≥ 60</td>
<td>20</td>
<td>24</td>
<td>0.922</td>
</tr>
<tr>
<td>Gender</td>
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<tr>
<td>Female</td>
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</tr>
<tr>
<td>Male</td>
<td>22</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Histological-type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epithelioid Cell</td>
<td>2</td>
<td>11</td>
<td>0.008*</td>
</tr>
<tr>
<td>Spindle Cell</td>
<td>20</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Spindle/Epithelioid Cell</td>
<td>17</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>Tumor basal diameter</td>
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<td></td>
<td>0.604</td>
</tr>
<tr>
<td>&lt;15 mm</td>
<td>9</td>
<td>11</td>
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</tr>
<tr>
<td>&gt;15 mm</td>
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<td>Pathologic-stage</td>
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</tr>
<tr>
<td>II</td>
<td>23</td>
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</tr>
<tr>
<td>III+IV</td>
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</tr>
<tr>
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<td>13</td>
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</tr>
<tr>
<td>No</td>
<td>36</td>
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<td></td>
</tr>
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<td>Dead</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>6</td>
<td>17</td>
<td>0.007*</td>
</tr>
<tr>
<td>No</td>
<td>34</td>
<td>23</td>
<td></td>
</tr>
</tbody>
</table>

Note: T: tumor, N: node, M: metastasis.
Expression of THBS2 in Cell Line

The RT-PCR analysis was conducted to detect THBS2 expression in UM cell lines, including SP6, M23, OCM-1A, and C918, and the normal UM cell line D78 (Figure 3). We found that there was no difference in the expression level of THBS2 in SP6 and OCM-1A cells, relative to normal UM cell line D78. However, THBS2 expression level in M23 and C928 cells was significantly higher than D78 cells ($p < 0.001$ for M23 cells, $p < 0.05$ for C928 cells). Thus, we used M23 cells for subsequent analysis.

Knockdown of THBS2 Inhibits Cell Proliferation and Growth of M23 Cells

With an attempt to reveal the role of THBS2 in M23 cells, the THBS2-specific si-THBS2 was transfected into M23 cells to determine its effect on cell proliferation and growth. The knockdown efficiency of THBS2 was analyzed using RT-PCR (A) and Western blotting (B and C) in M23 cells ($** p < 0.001$).

Table II. Univariate and multivariate analysis of clinical prognostic factors of UM.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$p$-value</td>
<td>HR</td>
</tr>
<tr>
<td>THBS2 expression</td>
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<td>4.651</td>
</tr>
<tr>
<td>Age</td>
<td>0.200</td>
<td>1.736</td>
</tr>
<tr>
<td>Gender</td>
<td>0.409</td>
<td>1.437</td>
</tr>
<tr>
<td>Histological-type</td>
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<td>0.338</td>
</tr>
<tr>
<td>Tumor basal diameter</td>
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<td>2.518</td>
</tr>
<tr>
<td>Pathologic-stage</td>
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<td>1.640</td>
</tr>
<tr>
<td>Pathologic-T</td>
<td>0.183</td>
<td>3.914</td>
</tr>
<tr>
<td>Pathologic-M</td>
<td>0.000*</td>
<td>27.462</td>
</tr>
<tr>
<td>Recurrence</td>
<td>0.001*</td>
<td>4.308</td>
</tr>
</tbody>
</table>

Figure 3. Relative expression levels of THBS2 in UM cell line including SP6, M23, OCM-1A, and C918 relative to normal melanocyte cell line (D78) cells. THBS2 expression was measured through RT-PCR and normalized to GAPDH. * $p < 0.05$, and ** $p < 0.001$.

Figure 4. Knockdown efficiency of THBS2 was analyzed using RT-PCR (A) and Western blotting (B and C) in M23 cells. ** $p < 0.001$.
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Figure 4. THBS2 level was examined using RT-qPCR and Western blotting 72 h after transfection (Figure 4). Figure 4 displayed that si-THBS2 transfected cells had a significant decrease both in mRNA and protein level of THBS2, when comparing with those in non-transfected cells ($p < 0.001$ both in RT-PCR and Western blotting).

Cell proliferation results demonstrated that the knockdown of THBS2 significantly inhibited the proliferation rate of M23 cells (Figure 5) at 48 h ($p < 0.05$), 72 h, 96 h ($p < 0.001$). Consistently, colony formation experiment also suggested that THBS2 knockdown remarkably suppressed the colony numbers of M23 cells ($p < 0.001$, Figure 6).

**THBS2 Knockdown Inhibits the Migratory/Invasive Ability**

After 24h scratch formation in the cell monolayer, the wound in the THBS2 knockdown cells was wider than that of the control cells (Figure 7A).

Then, transwell assays were performed to evaluate the potential effects of THBS2 knockdown on the invasive and migratory abilities. As listed in Figure 7B and C, invasive and migratory cell proportion in M23 cells after THBS2 knockdown was significantly lower than those in control cells ($p < 0.001$), implicating a suppression of invasive and migratory capacity of M23 cells after THBS2 expression level was inhibited.

**THBS2 Knockdown Inhibits the Activation of PI3K Signaling Pathway in M23 Cells**

To further reveal whether the dysregulated THBS2 expression regulates the metastasis, protein biomarkers including AKT, p-AKT, PI3K, p-PI3K, p70S6K were measured using Western blotting analysis.

Figure 8 showed that THBS2 knockdown significantly reduced the expression level of p-AKT, p-PI3K, and p70S6K in M23 cells ($p < 0.001$), but did not affect the total PI3K and AKT expression level.

Figure 5. Knockdown THBS2 in M23 cells significantly reduced the proliferative rate determined by CCK-8 assay. * $p < 0.05$, and ** $p < 0.001$.

Figure 6. Knockdown of THBS2 significantly reduced the colony-forming ability of M23 cells, evaluated using colony-formation assay. ** $p < 0.001$. 

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Discussion

Metastasis is an important hallmark of malignance, which is one of the most common poor prognostic factors in cancer patients. Many efforts have been done to identify molecular subtypes that can help indicate the prognosis of UM. Our study is different from previous studies that, to the best of our knowledge, this is the first study to evaluate the correlation between THBS2 level and clinical outcomes in UM to further investigate the potential mechanisms in UM metastasis. The GEO and TCGA results showed that over-expression of THBS2 was related to metastasis in UM patients, and these were confirmed by RT-PCR and Western blotting, which provides novel insights into this field of research through detecting THBS2 as a relevant biomarker of cancer metastasis.

Growing evidence has suggested that cell polarity, and cell migration may play important roles in advanced tumors and may be associated with the invasion into adjacent tissues and the formation of metastases. THBS2 is involved in cell adhesion, extracellular matrix (ECM) modeling, inflammatory responses, and angiogenesis. Moreover, Kim et al. has demonstrated that THBS2 can be applied for developing high specificity biomarkers sensing cancer invasion, and potential multi-cancer metastasis. In our study, knockdown of THBS2 inhibited the cell migration ability in M23 cells. In agreement with our study, THBS2 up-regulation was linked to poor prognosis in urothelial cancer, namely, disease-specific survival and metastasis-free survival. Thus, we believe that THBS2 overexpression is linked to poor prognosis, particularly through regulating the cell migration ability.

Cancer metastasis is a complicated and multi-step process. Essentially, the initial stage of metastatic progression is relying on a biological event referred to as epithelial-mesenchymal transition (EMT), which is characterized by loss of cell-cell adhesion, as well as enhanced cell motility. A study reported that activation of the PI3K/AKT pathway emerged as a key sig-
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PI3K/AKT signaling pathway in the regulation of EMT. PI3K/AKT is an important signaling pathway which modulates many cellular behaviors including cell survival, proliferation, migration/invasion, and metastasis. Once this pathway is activated, a downstream effector of PI3K, AKT, can be propagated to diverse substrates, for example, mammalian target of rapamycin (mTOR). Upon activated by mTOR, p70S6K directly phosphorylates S6 ribosomal protein, resulting in an increased proliferation of tumor cells. In the current study, THBS2 knockdown inhibited p-PI3K, p-AKT, p70S6K and suppressed cell proliferation, invasion, and migration. Hence, we infer that the inhibition of the PI3K/AKT pathway could account for the anti-metastatic effects of THBS2 in UM cell line M23.

Conclusions

We suggest that THBS2 is a valuable biomarker for UM prognosis. Furthermore, our study showed that THBS2 inhibited cell migration and invasiveness in human UM cells through inhibition of PI3K/AKT signaling pathway. Up-regulation of THBS2 was not only significantly related to aggressive clinicopathological parameters but also was an independent poor prognostic biomarker predicting shorter overall survival in UM patients. As evidenced by our results, THBS2 may be a powerful candidate for developing a preventive agent for UM metastasis.

Further studies are needed to advance our understanding of the involvement of THBS2 in UM development using an animal model, because this gene is a potential target for UM prevention and treatment.

Conflict of Interest

The Authors declare that they have no conflict of interest.

References


