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Clinical-Testis cancer

Preoperative plasma IGFBP2 is associated with nodal metastasis in patients with penile squamous cell carcinoma

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Abstract

Purpose: The nodal status is a strong predictor for clinical outcome in patients with penile cancer. We aimed to evaluate the association between preoperative plasma IGFBP2 levels and nodal status in patients with penile squamous cell carcinoma (PSCC).

Methods: This retrospective study enrolled 56 penile cancer patients who underwent penectomy between 2015 and 2017. Preoperative plasma IGFBP2 levels were detected by enzyme linked immunosorbent assay, which was analyzed in association with clinicopathological parameters (age, body mass index, pathological grade, phimosis, histological subtype, tumor stage, and nodal status). Univariable and Multivariable Cox regression analysis was conducted to identify the prognostic factors that influence disease free survival in PSCC. CCK8 assay and clonogenic assay were used to evaluate the cell viability and tumorigenic potential of PSCC cell line, respectively; wound healing assay, and transwell invasion assay were conducted to evaluate the effect of IGFBP2 depletion on cell migration and invasion in PSCC cells; IGFBP2 protein expression was analyzed by Western blotting.

Results: Plasma IGFBP2 levels were markedly higher in preoperative PSCC than those in healthy male subjects ($P = 0.0007$). Penectomy led to a significant reduction of plasma IGFBP2 levels in PSCC patients ($P = 0.0098$). Preoperative plasma IGFBP2 levels were significantly associated with nodal status of PSCC ($P < 0.0001$). At the cutoff value of 486.2 ng/ml, preoperative plasma IGFBP2 produced a sensitivity of 80.8% and a specificity of 86.7% to discriminate nodal metastasis. Preoperative plasma IGFBP2 levels could serve as independent prognostic factor for disease free survival in PSCC ($P = 0.001$). Further, knockdown of IGFBP2 expression suppressed cell growth, inhibited clonogenesis, and attenuated cell migration and invasion in Penl1 cells; depletion of IGFBP2 expression attenuated the levels of p-AKT and p-ERK1/2, while increased the expression of p16 and cleaved caspase-3 in Penl1 cells. Silencing IGFBP2 also led to a considerable decline of MMP2/9 levels in culture supernatant of Penl1 cells.

Conclusion: Higher preoperative plasma IGFBP2 was closely associated with nodal metastasis, which might serve as a useful diagnostic and prognostic biomarker for clinical management of PSCC. IGFBP2 might play an important role in the malignant progression of PSCC. Therapeutic strategies targeting IGFBP2-related signaling pathways may have a therapeutic benefit in PSCC patients.

Keywords: Penile cancer; IGFBP2; Nodal disease, Prognosis; Survival

1. Introduction

Penile squamous cell carcinoma (PSCC) accounts for the majority of penile cancer cases. PSCC is a rare malignancy in the United States (0.5–1.6 per 100,000 men), with a significantly higher incidence—up to 20 to 30 times greater—in areas of Africa and South America [1]. In some regions of Africa, South America, and Asia, PSCC can even account for up to 10% of cancers among men [1]. Despite advances in treatment, the prognosis of PSCC remains poor; a large-scale population-based investigation indicated that the survival of the patients with PSCC has not

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improved during the last 20 years [2]. The most important prognostic factor in PSCC is inguinal lymph node involvement [3]. Early resection definitely offers a survival benefit, but unfortunately, patients who undergo inguinal lymphadenectomy are prone to short- and long-term morbidity [4,5]. Although the therapeutic benefits outweigh these complications in patients with pathologic nodal involvement, only 20% of those with impalpable lymph nodes harbor occult metastasis [6,7]. Therefore, there is an urgent need to identify novel biomarkers with good sensitivity and specificity to detect the early nodal disease and improve clinical management of PSCC.

Serum/plasma biomarkers in cancer diagnosis or prognosis continue to be part of an evolving field and have been a promising noninvasive tool that may aid in the diagnosis and monitoring of disease in a number of different cancers. However, classical serum/plasma cancer biomarkers (such as Carcinoembryonic antigen, CA125, CA15-3, etc) are of no clinical value in PSCC [8–10]. Although squamous cell carcinoma antigen (SCC) levels seemed to correlate with tumor burden in patients with penile carcinoma, it lacks sensitivity in the detection of small tumor burdens and has little prognostic significance for survival after surgery [11]. The detection of lymph node metastases has been associated with the overexpression of p53 and Ki-67, as well as loss of E-cadherin, but these markers are not very useful in clinical practice [12].

Insulin-like growth factor-binding protein 2 (IGFBP2) is a member of a family of 6 highly conserved IGFBPs that are carriers for the insulin-like growth factors (IGFs). IGFBP2 contains RGD and HBD motifs which can directly bind to integrins and extracellular matrix and induce diverse biological actions independent of IGFs [13]. Recently, high serum/plasma IGFBP2 has been shown to be associated with poor clinical outcome in various solid malignancies, and is being evaluated as a potential cancer biomarker [14–19]. Herein, we aimed to evaluate the association between preoperative plasma IGFBP2 levels and nodal status in patients with PSCC.

2. Materials and methods

2.1. Patient and tumor characteristics

The 56 patients included in this retrospective study had complete patient and tumor characteristics, and underwent surgery for penile cancer between 2015 and 2017 at Xiangya Hospital ($n = 38$) and The Second Xiangya Hospital ($n = 18$), Central South University. Patients with known chemotherapy or brachytherapy before the surgery were excluded from the study. The clinical management of penile was conducted according to the NCCN guidelines for penile cancer [20]. Laparoscopic radical inguinal lymphadenectomy was performed after 2 to 4 weeks treatment of the primary penile cancer. Management of regional lymph nodes is stage dependent. For patients with clinically normal inguinal lymph nodes (cN0) pT1G3 and all higher stages were received radical inguinal lymphadenectomy. In clinically lymph node positive patients (cN1/cN2), surgical staging by radical inguinal lymphadenectomy is indicated. For patients with 2 or more positive lymph nodes, or 1 node with extracapsular extension (pN3), or fixed inguinal nodes (cN3), pelvic lymphadenectomy is indicated. Plasma samples of healthy male subjects ($n = 20$) were obtained from Yearth biomed Inc with informed consent. These healthy male subjects (median age 55, range 50–63) matched for the age of penile cancer patients must have no cancers or diseases such as acute infection, diabetes, and ischemia. TNM staging was assigned based on the American Join Committee on Cancer, eighth edition [21]. Penile cancer tissues and matched adjacent noncancerous penile tissues ($n = 7$) were also collected for Western blotting analysis. The study protocols were approved by the research ethics committee in Xiangya hospital (Rev No. 201805847) with informed consent having been obtained from all patients. Patient and tumor characteristics obtained from our computerized institutional databases included age, stage, regional lymph node involvement or distant metastasis, histological subtype, tumor grade, and body mass index (BMI) as well as phimosis, etc. All patients were prescribed a follow-up regimen based on the National Comprehensive Cancer Network guidelines, with physical examination every 3 to 6 months depending on nodal stage. Cancer and vital status were determined by both clinical follow-up at our institution (median follow-up: 26 months).

2.2. Reagents and cell line

Antibodies against IGFBP2 and β-actin were obtained from Abcam (Cambridge, MA); Antibodies against p-AKT, AKT, p53, p-ERK1/2, ERK1/2, p16, and Cleaved Caspase-3 were purchased from Cell Signaling Technology (Beverly, MA); Human PSCC cell line Penl1 was kindly provided by Prof. Hui Han (Department of Urology, Cancer hospital, Sun Yat-sen University) [22]. Penl1 was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 4 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Lentiviral plasmids expressing scramble or shIGFBP2 was purchased from Genecopoeia Inc. (Rockville, MD). The packaging procedure for lentiviral shRNAs was conducted as we described previously [23,24].

2.3. Clonogenic survival assay

Clonogenic survival assay was used to measure the tumorigenic potential of Penl1 cells [25]. Briefly, Penn1 cells were plated in 6 cm culture dishes, and cultured for 12 days, and the number of surviving colonies (defined as a colony with >50 cells) was stained with 0.5% crystal violet and counted.

2.4. Cell viability analysis

Cell viability was determined by CCK-8 assay (Dojindo Laboratories, Kumamoto, Japan) as described previously [26]. Briefly, Penl1 cell were seeded at a concentration of 1×10^4 cells/200 μl/well into 96-well plates, and allowed an overnight period for attachment. After different time point in culture, and 10 μl CCK-8 solution was added and cells were incubated for 1 h at 37°C. The optical density (OD) value (absorbance) was measured at 450 nm by a Infinite F50 microplate reader (Tecan, Switzerland).

2.5. Western blot

Western blot was conducted as described previously [27]. Briefly, cells were lysed and quantified using Bicinchoninic Acid Protein Assay Kit (Beyotime Biotechnology, Shanghai, China). Protein lysates (15 μg) was separated by 10% SDS-PAGE and transferred to Hybond-P PVDF membranes (Amersham Biosciences). Blots were blocked with 5 % nonfat dry milk in tris-buffered saline buffer for 2 hours at room temperature and then incubated with diluted antibodies overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated secondary antibody (Abcam, Cambridge, MA) for 1 hours at room temperature. The signal was visualized with an enhanced chemiluminescence detection reagent (Abcam, Cambridge, MA). β-actin served as a loading control.

2.6. Wound healing assay

Cell migration was evaluated using wound healing assay as described previously [28]. For cell migration assay, Penl1 cells (5×10^5) were grown in 6-well plates for 48 hours until the cells were fully confluent. After nonadherent cells were washed away twice with PBS, a sterile 200 μl pipette tip was used to make a uniform scratch in the center of the well. After 0 and 24 hours, the distance between the wound sides was measured.

2.7. Transwell invasion assay

Cell invasion assay were performed based on transwell chamber with 8 μm pores (Corning) as described previously [29]. Briefly, Penl1 cells were suspended in DMEM medium at 5×10^5 cells/ml. Each transwell of 24-well plate was precoated with 50 μl Matrigel. RPMI 1640 medium (600 μl) containing 10% FBS was added to each well (lower compartment), and 0.1 ml (0.5×10^5 cells) of cell suspension was added onto each transwell insert (upper compartment). The plates were incubated for 36 hours at 37°C. The invaded cells on the bottom surface of the membrane were fixed by dehydrated alcohol, and stained by 0.2% crystal violet solution (Sigma-Aldrich). After the wash, cells were photographed with Olympus BX43 microscope. Then the stained cells were eluted by 20% glacial acetic acid and measured with a microplate reader (Tecan, Switzerland) at 570 nm.

2.8. Enzyme linked immunosorbent assay

For the analysis of IGFBP2, blood plasma samples had been collected 1 day before the operation and postoperative day 28. The blood samples were centrifuged at 3500 rpm for 15 minutes. The supernatant was aliquoted and then stored at −80°C until use. The enzyme linked immunosorbent assay (ELISA) method was used to measure the IGFBP2 levels in plasma according to the manufacturer's protocol (RayBiotech, Norcross, GA). For MMP2 and MMP9 analysis, cell culture supernatant was centrifuged at 12,000 g for 15 minutes at 4°C. MMP2 ELISA kits (Sigma) and MMP9 ELISA kits (Sigma) were used to measure the levels of protein secretion of MMP2 and MMP9 by Penl1 cells, respectively, according to the manufacturer's instructions [30].

2.9. Statistical analysis

SPSS 16.0 was used for statistical analysis. Mann-Whitney tests were applied to compare the levels of preoperative plasma IGFBP2 of 2 groups. Kruskal-Wallis test followed by a Dunn's post-test was performed to compare the levels

Table 1
Clinicopathologic characteristic of patient cohort

Parameters	Cases (%)
Age (year)	
≤54	24 (42.9%)
>54	32 (57.1%)
Body mass index (kg/m2)	
≥24	27 (48.2%)
<24	29 (51.8%)
Phimosis	
Yes	51 (91.1%)
No	5 (8.9%)
pT stage	
Ta/T1	31 (55.4%)
T2	25 (44.6%)
Grade	
Low	2 (3.6%)
Intermediate	14 (25.0%)
High	40 (71.4%)
Histological subtype	
Usual	42 (75.0%)
Papillary	1 (1.8%)
Warty	7 (12.5%)
Verrucous	6 (10.7%)
Nodal metastasis	
Negative (N0)	30 (53.6%)
Positive (N1)	26 (46.4%)
Penile Surgery	
Penile preservation	20 (35.7%)
Partial penectomy	35 (62.5%)
Radical penectomy	1 (1.8%)
Inguinal lymphadenectomy	
No	24 (42.9%)
Yes	32 (57.1%)
Pelvic lymphadenectomy	
No	53 (94.6%)
Yes	3 (5.4%)

of preoperative plasma IGFBP2 in 3 or more groups. Wilcoxon rank sum test was conducted to compare the pre- and postoperative plasma IGFBP2 levels. Receiver operating characteristic curves (sensitivity plotted against 1-specificity) were constructed and the area under the curve (AUC) was calculated as a comparative measure of diagnostic accuracy for nodal metastasis. The optimal IGFBP2 cut-off values for prognosis prediction were calculated using receiver-operating characteristic curve analysis with reference to cancer recurrence [31]. Multivariable Cox regression analysis was conducted to identify the prognostic factors that influence disease free survival (DFS). A 2-tailed $P < 0.05$ was considered significant in all tests.

3. Results

3.1. IGFBP2 levels were elevated in plasma and cancer tissues of preoperative PSCC

A total of 56 men who suffered from SCC of the penis and underwent penile cancer surgery were retrospectively reviewed with a median age of 57 (34–79) years. Of these, 20 (35.7%) whose treatment plan was penile preservation underwent local excision with circumcision, 35 (62.5%) underwent partial penile amputation, and 1 (1.8%) underwent total penile amputation. The total patient population had a mean BMI of 23.43 kg/m^2 (23.43 ± 2.18 kg/m^2) prior to surgery. Twenty five of all patients (44.6%) suffered from locally advanced penile cancer (≥pT2); nodal disease was seen in 26 cases (46.6%) at the time of surgery. Thirty two cases (57.1%) had conducted lymphadenectomy during the surgery. Table 1 gives a detailed summary of patient and tumor characteristics, including stage and grade.

To assess the diagnostic potential of IGFBP2, plasma levels were determined in healthy donors and PSCC. Plasma IGFBP2 was significantly elevated in preoperative PSCC cases (579.92 ± 357.59 ng/ml, mean ± standard deviation) when compared with plasma from healthy donors (329.12 ± 128.42 ng/ml, $P = 0.0007$; Fig. 1A). Further, plasma IGFBP2 was significantly reduced in postoperative cases (354.63 ± 59.81 ng/ml) when compared with matched preoperative PSCC cases (557.14 ± 215.88 ng/ml, $P = 0.0098$; Fig. 1B). Western blotting analysis showed that IGFBP2 was mostly higher in cancer tissues than in matched adjacent noncancerous tissues (Fig. 1C).

3.2. Preoperative plasma IGFBP2 levels were associated with nodal metastasis and unfavorable disease free survival

To examine the correlation of biomarker with clinicopathological parameters of disease, preoperative plasma IGFBP2 levels were analyzed in association with age, BMI, pathological grade, phimosis, histological subtype, tumor stage, and nodal status. Preoperative plasma IGFBP2 levels were significantly higher in patients with nodal diseases (Fig. 2A, $P < 0.0001$). Preoperative IGFBP2 plasma levels were not significantly different when compared by age

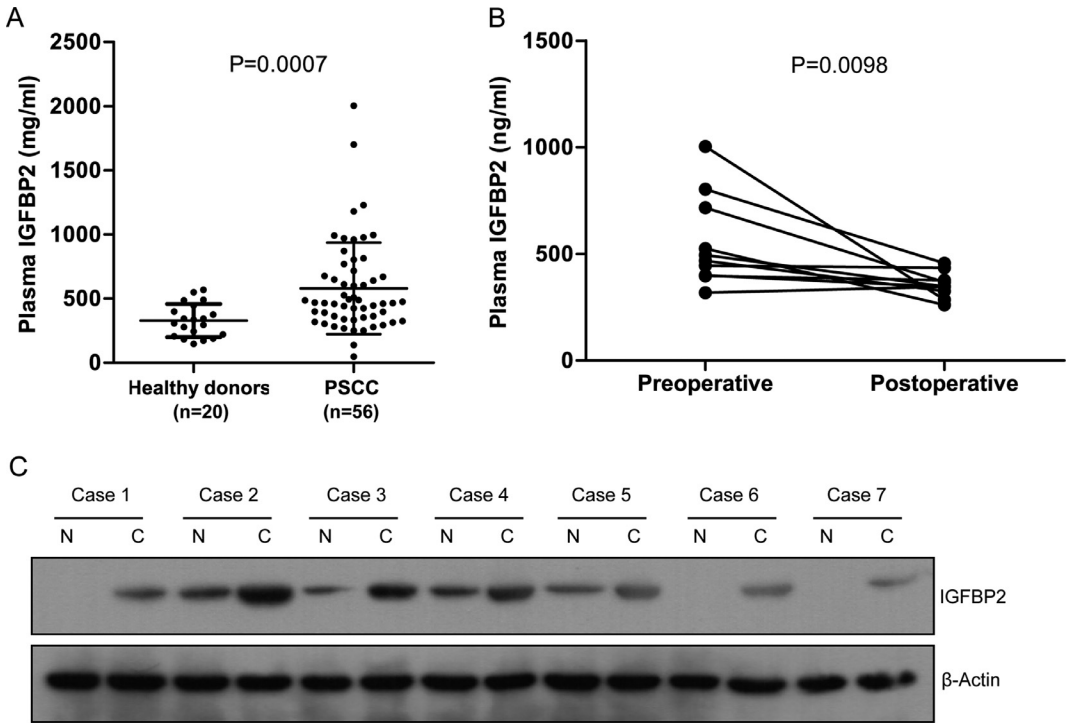

Figure 1. (A) Plasma IGFBP2 levels in preoperative PSCC cohort (n =56) and healthy male subjects (n =20). (B) Plasma IGFBP2 levels in matched preoperative/postoperative PSCC cohort (n =10). (C) IGFBP2 protein expression in matched cancerous and adjacent non-cancerous tissues (n =7). β-actin served as a loading control.

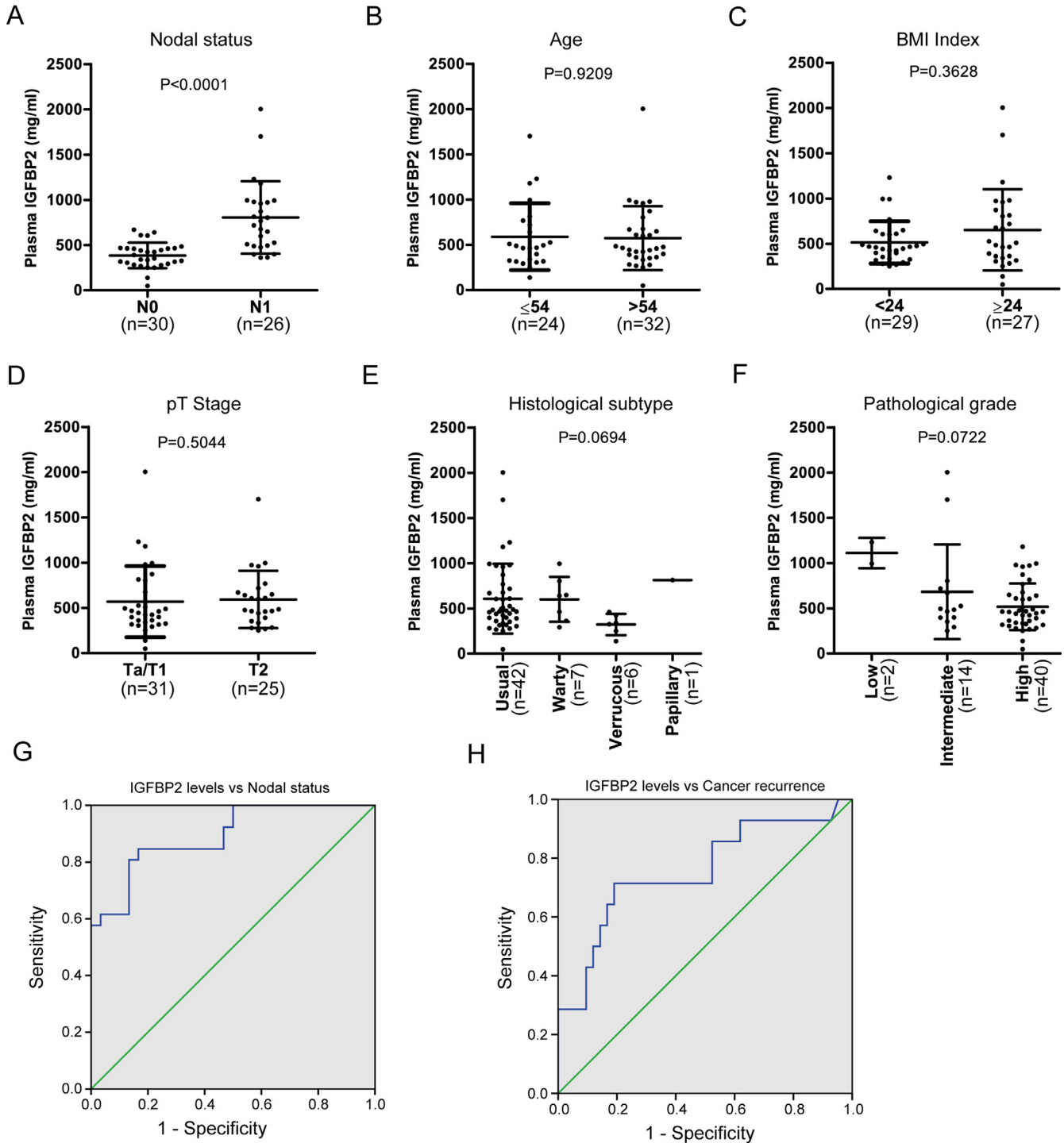

Figure 2. Correlation of preoperative plasma IGFBP2 levels with clinicopathologic parameters. (A) Nodal status; (B) Age; (C) BMI index; (D) pT stage; (E) pathological grade; (F) Histological subtype. (G) Receiver-operating characteristics (ROC) curves for IGFBP2 with reference to nodal status. (H) Receiver-operating characteristics (ROC) curves for IGFBP2 with reference to cancer recurrence.

($P = 0.9209$), pathological grade ($P = 0.0722$), histological subtype ($P = 0.0694$), BMI ($P = 0.3628$), and pT tumor stage ($P = 0.5044$) (Fig. 2B–F). Based on receiver operating characteristic analysis, at the cutoff value of 486.2 ng/ml, IGFBP2 produced a sensitivity of 80.8% and a specificity of 86.7% to discriminate nodal metastasis (AUC = 0.892, $P <$ 0.0001; Fig. 2G). Moreover, at the cutoff value of 625.2 ng/ml, IGFBP2 produced a sensitivity of 71.4% and a specificity of 81.0% to discriminate cancer recurrence (AUC = 0.756, $P = 0.004$, Fig. 2H). On Kaplan-Meier survival analysis, both nodal metastasis ($P = 0.017$) and higher plasma IGFBP2 ($P = 0.000$) were associated with shorter

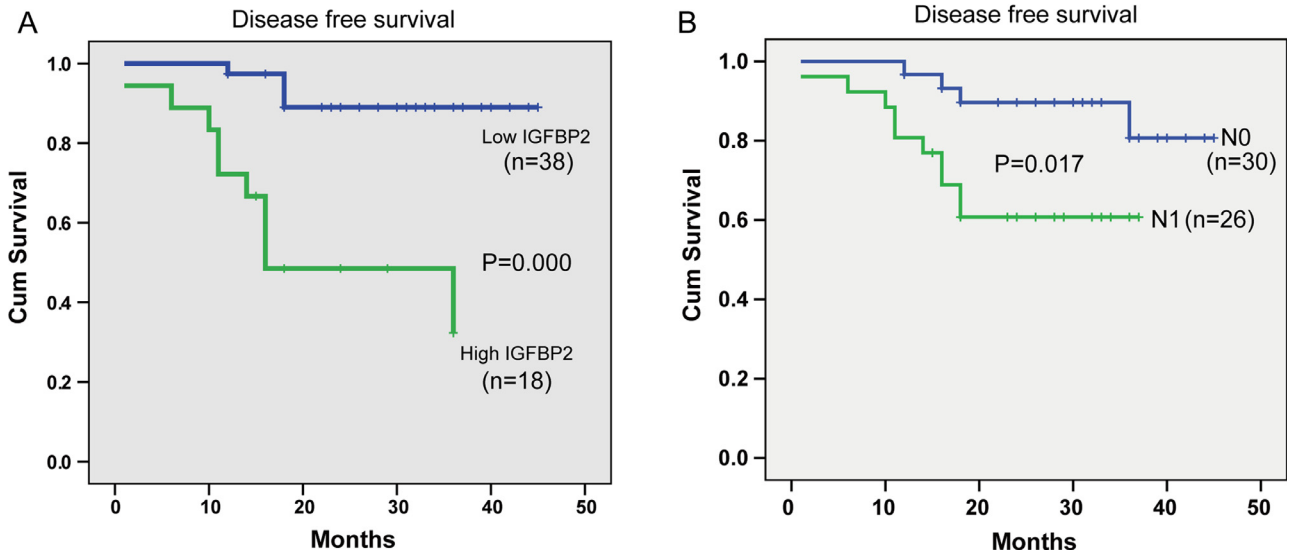

Figure 3. Higher preoperative plasma IGFBP2 levels was associated with unfavorable disease free survival (DFS) in PSCC (n =56). Survival analysis was performed using the Kaplan-Meier method to determine DFS based on IGFBP2 cut-off value (625.2 ng/ml) (A) or nodal status (B).

DFS (Fig. 3). Univariable Cox regression analysis results showed that pathological grade, nodal status, and higher preoperative plasma IGFBP2 levels were prognostic factors for DFS in PSCC (Table 2). However, Multivariable Cox regression analysis indicated that only pathological grade ($P = 0.028$) and higher preoperative plasma IGFBP2 levels ($P = 0.001$) could be independent prognostic factor for shorter DFS (Table 2).

3.3. IGFBP2 promotes cell growth and clonogenesis in Penl1 cells

We sought to further investigate the oncogenic function of IGFBP2 in PSCC using Penl1 as cell model. Penl1 cells were transduced with nontargeting scramble (Scr) or specific shRNA targeting IGFBP2 (shIGFBP2). As shown in Fig. 4A, IGFBP2 expression was significantly reduced in Penl1 cells transduced with shIGFBP2 lentivirus, whereas it was not significantly affected by Scr shRNA. We next evaluated the effect of IGFBP2 expression on the cell growth of Penl1 cells by CCK-8 assay, and the results showed that shIGFBP2 transduced-Penl1 cells (cell doubling time: 48.3± 3.5 hours) grew slower than those transduced with Scr shRNA (cell doubling time: 32.3 ± 2.5 hours) ($P = 0.0077$; Fig. 4B). Furthermore, colony formation of Penl1 cells in shIGFBP2 group (64.2 ± 10.1 %) decreased greatly, as compared with Scr group (100.0 ± 4.1 %), ($P = 0.0029$; Fig. 4C).

3.4. Depletion of IGFBP2 expression attenuates cell migration and invasion in Penl1 cells

The metastatic process involves cell scattering, motility, ECM degradation, migration, and invasion through the basement membranes [32]. Since overexpression of IGFBP2 is correlated with lymph node metastasis and unfavorable progression free survival, we proposed that IGFBP2 might regulate the migration/invasion phenotypes in PSCC. To examine whether IGFBP2 affects PSCC cancer cell migration in vitro, we performed wound-healing experiments using Penl1 cells and measured the distance of cell migration to the wound area. We observed a significantly slower wound-healing rate in the cells expressing shIGFBP2 shRNA (28.5 ± 3.2 μm/hour) compared to the control Scr cells (47.0 ± 5.9 μm/hour) ($P = 0.0227$, Fig. 5A). To examine the effects of IGFBP2 on cell invasion, transwell invasion assay was performed. As

Table 2
Cox univariate and multivariate proportional hazard model for factors affecting disease-free survival in PSCC cases (n = 56)

	Univariate analysis		Multivariate analysis	
	HR(95%CI)	P value	HR(95%CI)	P value
pT stage		0.316		0.500
Pathological grade	3.782 (1.308-10.935)	0.014	0.293 (0.098-0.874)	0.028
Nodal status	3.708 (1.153-11.924)	0.028		0.221
IGFBP2	7.685 (2.394-24.670)	0.001	0.138(0.042-0.447)	0.001

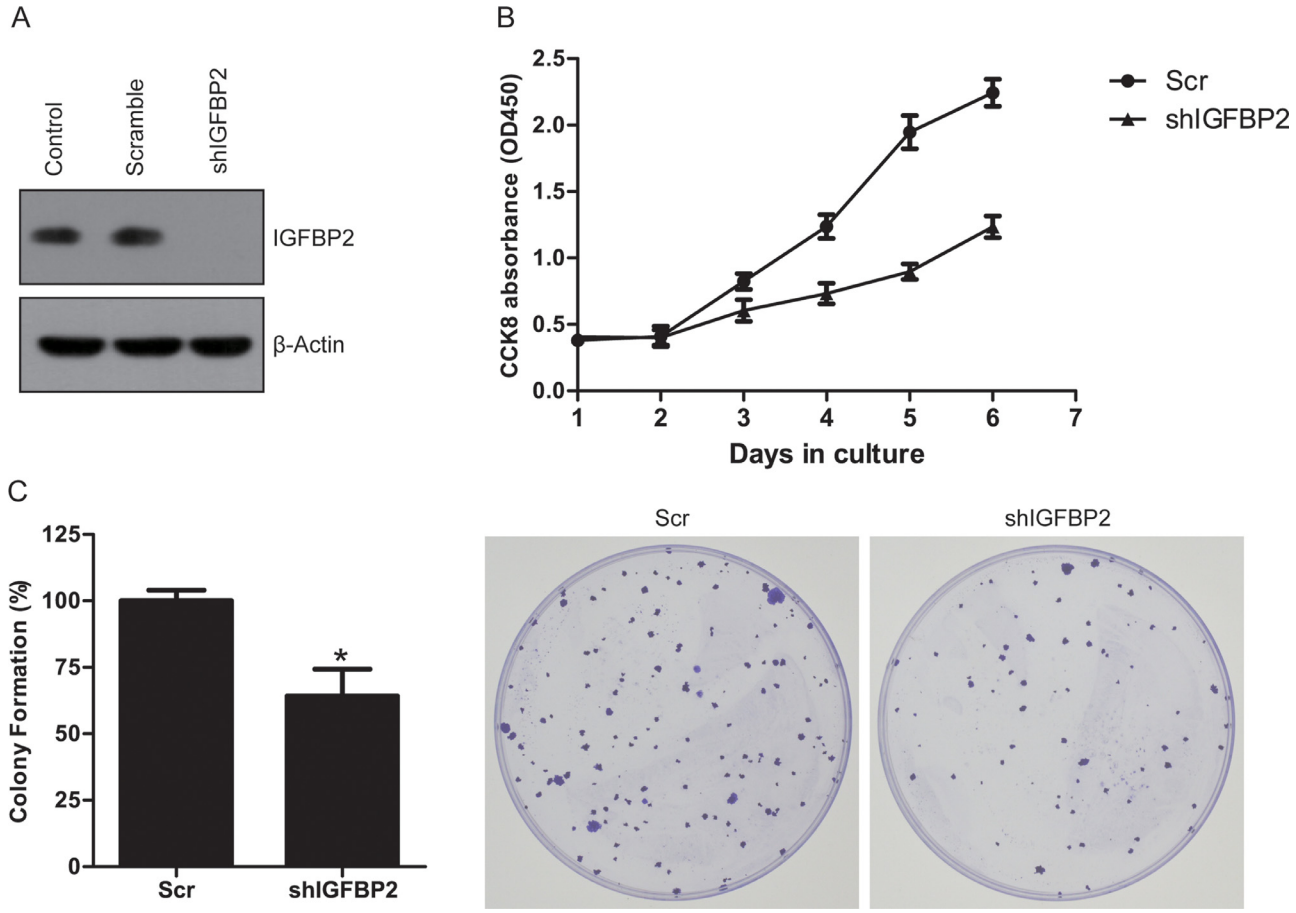

Figure 4. Knockdown of IGFBP2 expression suppresses cell growth and clonogenesis in Penl1 cells. (A) IGFBP2 expression was significantly reduced in Penl1 cells transduced with shIGFBP2 lentivirus. β-actin served as a loading control. (B) Knockdown of IGFBP2 expression attenuated cell growth of Penl cells. The CCK-8 absorbance was measured at 450 nm (OD_{450}). n =3, *$P < 0.05$, Scr vs. shIGFBP2. (C) Depletion of IGFBP2 expression reduced clonogenesis of Penl1 cells. The colony formed in scramble control (Scr) was regards as 100%. Unpaired t test with Welch's correction, n =3, *$P = 0.0029$, Scr vs. shIGFBP2. All experiments were performed 3 times, and data are presented as mean±S.D. values. *$P < 0.05$, Scr vs. shIGFBP2.

shown in Fig. 5B, knockdown of IGFBP2 expression attenuated the invasion of Penl1 cells (OD_{570}: 0.324 ± 0.034), as compared with Scr control (OD_{570}: 0.739 ± 0.028) ($P = 0.0005$).

3.5. *IGFBP2 regulates cancer-related signaling pathways in Penl1 cells*

Currently, little is known about the molecular mechanisms of carcinogenesis of PSCC; HPV infection, mutations in p53 and H-Ras, and dysregulated pathways such as EGFR, PI3K/AKT/mTOR, and p16 have been identified as potential cancer-related genes/pathways involved in PSCC [33–35]. As shown in Fig. 6A, knockdown of IGFBP2 attenuated p-AKT and p-ERK1/2, while increased tumor suppressor p16 and apoptotic molecule cleaved caspase-3. Furthermore, ELISA assay revealed that depletion of IGFBP2 reduced secretion of 2 invasion/metastasis-related molecules MMP2 (16.3 ± 2.5 ng/ml) and MMP9 (25.5 ± 4.6 ng/ml), as compared with Scr control (MMP2: 60.2 ± 5.1 ng/ml, $P = 0.0055$; MMP9: 48.3 ± 2.7 ng/ml, $P = 0.0050$) (Fig. 6B).

4. Discussion

IGFBP2 is one of the most commonly and abundantly expressed IGFBPs in a broad range of human cancers [36]. Serum/plasma IGFBP2 levels positively correlate with tumor grade, aggressiveness, and metastasis in malignancy including high grade glioma, pancreatic, breast, and prostate cancer [37–40]. Consistent with IGFBP2 studies in aforementioned cancers, we showed that plasma IGFBP2 levels were markedly higher in preoperative PSCC than those in healthy male subjects. We also observed that plasma IGFBP2 was significantly reduced in postoperative cases when compared with matched preoperative PSCC cases, suggesting high plasma IGFBP2 levels might be caused by the tumor burden in PSCC patients. Preoperative plasma IGFBP2 levels were significantly associated with nodal status of PSCC. At the cutoff value of 486.2 ng/ml, preoperative plasma IGFBP2 produced a sensitivity of 80.8% and a specificity of 86.7% to discriminate nodal metastasis. Our Cox multivariable analysis showed that high IGFBP2 levels (>625.2 ng/ml) could serve as a

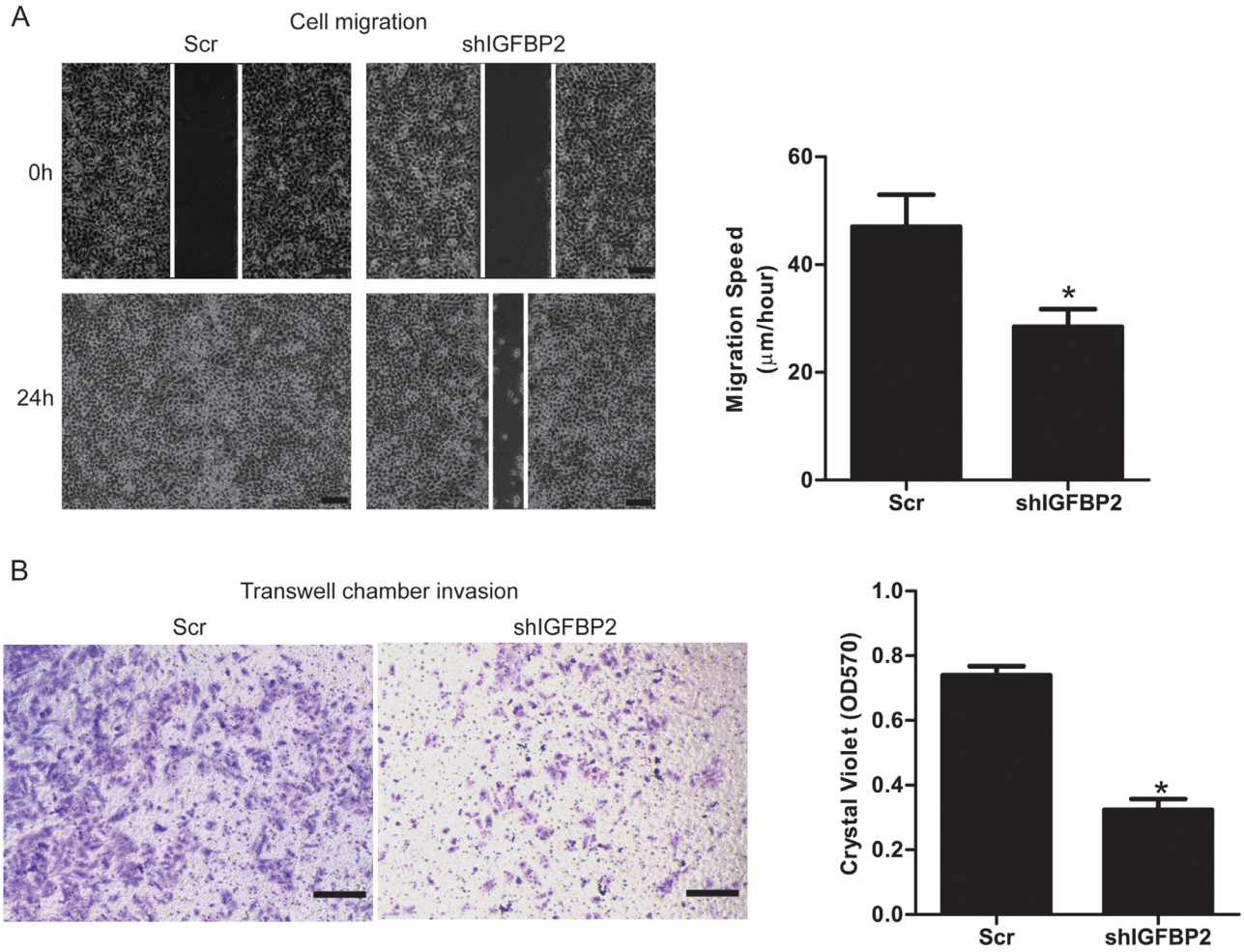

Figure 5. Knockdown of IGFBP2 expression inhibits cell migration and invasion in Penl1 cells. (A) Wound healing Assay. The migration of the cells to the wound was visualized with an inverted Olympus phase-contrast microscope at 0 and 24 hours after scratch. The representative fields were photographed; the relative healing rates were quantified with measurements of the gap sizes after the culture. Three different areas in each assay were chosen to measure the distances of migrating cells to the origin of the wound. Bars: 100 μm. Unpaired t test with Welch's correction, $^*P < 0.05$, Scr vs. shIGFBP2. (B) Transwell invasion assays with Penl1 cells were performed in scramble control and shIGFBP2group. Cell migration and invasion capability were quantified by crystal violet assay (OD_{570}). Bars: 100 μm. All experiments were performed 3 times, and data are presented as mean ± S.D. values. Unpaired *t* test with Welch's correction, $n = 3$, $^*P < 0.05$, Scr vs. shIGFBP2.

potential prognostic marker for PSCC. These results might reflect the potential value of preoperative plasma IGFBP2 levels as a useful diagnostic and prognostic biomarker for clinical management of PSCC. However, our conclusions need to be validated in a larger and independent PSCC cohort as the cohort in the present study is relatively small.

The path of cancer progression is determined by alterations in the regulatory mechanisms of proliferation and migration/invasion. The key molecular alterations driving PSCC development and potential therapeutic targets are incompletely understood, although significant advances have been made in characterizing the molecular pathways driving carcinogenesis in penile cancer. IGFBP2 plays a key role in the proliferation and survival of cancer cells, pointing to a crucial role for IGFBP-2 in mitogenic and survival capacity of these cancer models [36]. Cell line models could be used to investigate IGFBP2 role in tumorigenesis of PSCC, since our findings revealed that IGFBP2 was potentially linked with nodal metastasis and unfavorable prognosis in PSCC. Consistently, our findings indicated that IGFBP2 might play an important role in promoting the tumorigenesis and malignant phenotype of PSCC cells, as knockdown of IGFBP2 expression suppressed cell growth, inhibited clonogenesis, and attenuated cell migration and invasion in Penl1 cell model. Therefore, a better understanding of the cellular and molecular mechanisms activated or regulated by IGFBP2 could ultimately improve our understanding of tumor initiation and malignant progression of PSCC.

In a series of experiments in PSCC tissues and in cell line model, we showed that decreased expression of IGFBP2 was associated with a down-regulation of proliferation, migration and/or invasion of Penl1 cells. Our findings also revealed that knockdown of IGFBP2 attenuated p-AKT

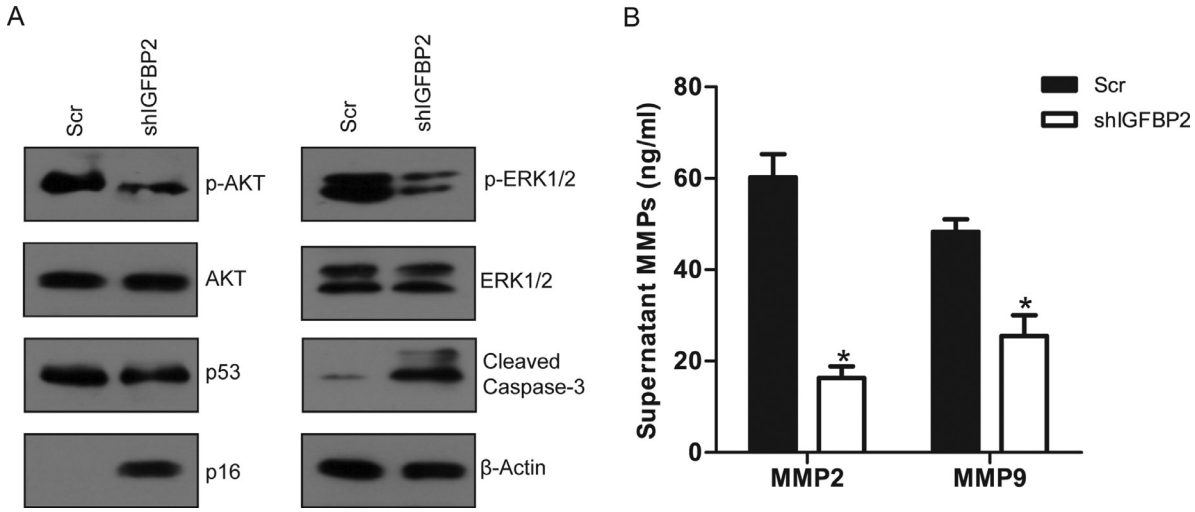

Figure 6. (A) Knockdown of IGFBP2 expression affected p-AKT, p-ERK1/2, p16, and cleaved caspase-3 expression. β-actin served as a loading control. (B) Knockdown of IGFBP2 expression reduced MMP2/9 secretion in Penl1 cells. All experiments were performed 3 times, and data are presented as mean ± S. D. values. Unpaired t test with Welch's correction, n =3, *$P < 0.05$, Scr vs. shIGFBP2.

and p-ERK1/2, increased tumor suppressor p16 and cleaved caspase-3, and reduced metastasis-related marker MMP2/9 levels. As activation of AKT pathway and p16 status has already been demonstrated to be crucial for the tumorigenesis of PSCC [41,42], it would be reasonable to propose that the effects of IGFBP2 might result from its function in regulating the balance of oncogene/tumor suppressor genes, enhancing the expression of metastasis-related molecules, thus promoting the tumor progression of PSCC.

Although our study is the first to show the association between preoperative plasma IGFBP2 and nodal metastasis in PSCC, we were conscious of the potential limitations of this study. First, our study was a retrospective study. Chinese patients with metastatic penile cancer are typically poor and undereducated and live in rural regions affected by selection bias, which is inherent to retrospective studies. Second, the follow-up period was relatively short; therefore, longer follow-up periods are needed. Third, the diversity of treatment in the present study, that is, pelvic lymphadenectomy, may potentially affect other variables such as patient survival. Although pelvic lymphadenectomy was recommended by the guidelines as standard treatment for locally advanced penile cancer, some patients refused because of socioeconomic reason, which potentially adds biases. Fourth, the limits of small cohort (n = 56) enrolled in this study. The limited number of patients and other limitations described above, however, also inhibited our ability to perform multivariate analyses and to select patients for inguinal lymph node dissection vs. surveillance. Therefore, larger PSCC cohort and prospective study would be warranted in order to further elucidate the usefulness of IGFBP2 as a biomarker for the prediction of prognosis or nodal status in PSCC. Overall, the relevance of preoperative plasma IGFBP2 examined in the present study and their potential usefulness in patients should be validated externally using larger, independent datasets.

5. Conclusion

In conclusion, we have shown that high preoperative plasma IGFBP2 levels are closely associated with nodal metastasis and might serve as diagnostic and prognostic biomarker for clinical management of PSCC. Further, IGFBP2 might play an important role in promoting the malignant phenotype of PSCC cells. These results would highlight the importance of a potential regulator in the aggressiveness and spread of PSCC, and suggest that IGFBP2 expression could be a valuable therapeutic target for patients with penile cancers. We will next expand our study cohort in order to confirm the usefulness of preoperative plasma IGFBP2 as a diagnostic and prognostic marker in PSCC. If confirmed in additional cohorts, preoperative plasma IGFBP2 levels may be a useful prognostic biomarker adjunct to traditional histopathological staging. Future prospective clinical study would be required to assess the possibility whether preoperative plasma IGFBP2 could serve as a useful marker to decide the effectiveness and cost-effectiveness of the lymphadenectomy as a therapeutic approach to improve survival.

References

[1] Douglawi A, Masterson TA. Updates on the epidemiology and risk factors for penile cancer. Transl Androl Urol 2017;6:785–90.
[2] Barski D, Georgas E, Gerullis H, et al. Metastatic penile carcinoma – an update on the current diagnosis and treatment options. Cent Eur J Urol 2014;67:126–32.
[3] Ficarra V, Novara G, Boscolo-Berto R, et al. How accurate are present risk group assignment tools in penile cancer? World J Urol 2009;27:155–60.
[4] Kroon BK, Horenblas S, Lont AP, et al. Patients with penile carcinoma benefit from immediate resection of clinically occult lymph node metastases. J Urol 2005;173:816–9.

[5] Bevan-Thomas R, Slaton JW, Pettaway CA. Contemporary morbidity from lymphadenectomy for penile squamous cell carcinoma: the M. D. Anderson Cancer Center Experience. J Urol 2002;167:1638–42.
[6] Solsona E, Algaba F, Horenblas S, et al. EAU guidelines on penile cancer. Eur Urol 2004;46:1–8.
[7] Ornellas AA, Seixas AL, Marota A, et al. Surgical treatment of invasive squamous cell carcinoma of the penis: retrospective analysis of 350 cases. J Urol 1994;151:1244–9.
[8] Grunnet M, Sorensen JB. Carcinoembryonic antigen (CEA) as tumor marker in lung cancer. Lung Cancer 2012;76(May (2)):138–43.
[9] Felder M, Kapur A, Gonzalez-Bosquet J, et al. MUC16 (CA125): tumor biomarker to cancer therapy, a work in progress. Mol Cancer 2014;13(May (29)):129.
[10] Li X, Dai D, Chen B, et al. Clinicopathological and prognostic significance of cancer antigen 15-3 and carcinoembryonic antigen in breast cancer: a meta-analysis including 12,993 patients. Dis Markers 2018;2018(May (2)):9863092.
[11] Zhu Y, Ye DW, Yao XD, et al. The value of squamous cell carcinoma antigen in the prognostic evaluation, treatment monitoring and followup of patients with penile cancer. J Urol 2008;180:2019–23.
[12] Zhu Y, Zhou XY, Yao XD, et al. The prognostic significance of p53, Ki-67, epithelial cadherin and matrix metalloproteinase-9 in penile squamous cell carcinoma treated with surgery. BJU Int 2007;100:204–8.
[13] Akmal SN, Yun K, MacLay J, et al. Insulin-like growth factor 2 and insulin-like growth factor binding protein 2 expression in hepatoblastoma. Hum Pathol 1995;26:846–51.
[14] Sehgal P, Kumar N, Praveen Kumar VR, et al. Regulation of protumorigenic pathways by insulin like growth factor binding protein2 and its association along with β-catenin in breast cancer lymph node metastasis. Mol Cancer 2013;12:63.
[15] Mehrian-Shai R, Chen CD, Shi T, et al. Insulin growth factor-binding protein 2 is a candidate biomarker for PTEN status and PI3K/Akt pathway activation in glioblastoma and prostate cancer. Proc Natl Acad Sci USA 2007;104:5563–8.
[16] Moore LM, Holmes KM, Smith SM, et al. IGFBP2 is a candidate biomarker for Ink4a-Arf status and a therapeutic target for high-grade gliomas. Proc Natl Acad Sci USA 2009;106:16675–9.
[17] Russell MR, Graham C, D'Amato A, et al. A combined biomarker panel shows improved sensitivity for the early detection of ovarian cancer allowing the identification of the most aggressive type II tumours. Br J Cancer 2017;117:666–74.
[18] Wang H, Arun BK, Wang H, et al. IGFBP2 and IGFBP5 overexpression correlates with the lymph node metastasis in T1 breast carcinomas. Breast J 2008;14:261–7.
[19] Resovi A, Bani MR, Porcu L, et al. Soluble stroma-related biomarkers of pancreatic cancer. EMBO Mol Med 2018 Aug;10(8):pii: e8741.
[20] Clark PE, Spiess PE, Agarwal N, et al. Penile cancer: clinical practice guidelines in oncology. J Natl Compr Canc Netw 2013;11:594–615.
[21] Paner GP, Stadler WM, Hansel DE, Montironi R, Lin DW, Amin MB. Updates in the eighth edition of the tumor-node-metastasis staging classification for urologic cancers. Eur Urol 2018;73:560–9.
[22] Chen J, Yao K, Li Z, et al. Establishment and characterization of a penile cancer cell line, penl1, with a deleterious TP53 mutation as a paradigm of HPV-negative penile carcinogenesis. Oncotarget 2016;7:51687–98.
[23] Liu Q, Zhang C, Yuan J, et al. PTK7 regulates Id1 expression in CD44-high glioma cells. Neuro Oncol 2015;17:505–15.
[24] Jun F, Hong J, Liu Q, et al. Epithelial membrane protein 3 regulates TGF-β signaling activation in CD44-high glioblastoma. Oncotarget 2017;8:14343–58.
[25] Yang Y, Huang JQ, Zhang X, et al. MiR-129-2 functions as a tumor suppressor in glioma cells by targeting HMGB1 and is down-regulated by DNA methylation. Mol Cell Biochem 2015;404:229–39.
[26] Li P, Tian W, Ma X. Alpha-mangostin inhibits intracellular fatty acid synthase and induces apoptosis in breast cancer cells. Mol Cancer 2014;13:138.
[27] Zhang J, Fu J, Pan Y, et al. Silencing of miR-1247 by DNA methylation promoted non-small-cell lung cancer cell invasion and migration by effects of STMN1. Onco Targets Ther 2016;9:7297–307.
[28] Yang C, Xu Y, Cheng F, et al. miR-1301 inhibits hepatocellular carcinoma cell migration, invasion, and angiogenesis by decreasing Wnt/β-catenin signaling through targeting BCL9. Cell Death Dis 2017;8:e2999.
[29] He J, Xu Q, Wang M, et al. Oral administration of apigenin inhibits metastasis through AKT/P70S6K1/MMP-9 pathway in orthotopic ovarian tumor model. Int J Mol Sci 2012;13:7271–82.
[30] Zhang JF, Wang P, Yan YJ, et al. IL-33 enhances glioma cell migration and invasion by upregulation of MMP2 and MMP9 via the ST2-NF-κB pathway. Oncol Rep 2017;38:2033–42.
[31] Li ZS, Yao K, Li YH, et al. Clinical significance of preoperative C-reactive protein and squamous cell carcinoma antigen levels in patients with penile squamous cell carcinoma. BJU Int 2016;118(Aug (2)):272–8.
[32] Shan N, Shen L, Wang J, et al. MiR-153 inhibits migration and invasion of human non-small-cell lung cancer by targeting ADAM19. Biochem Biophys Res Commun 2015;456:385–91.
[33] Chaux A, Munari E, Katz B. The epidermal growth factor receptor is frequently overexpressed in penile squamous cell carcinomas: a tissue microarray and digital image analysis study of 112 cases. Hum Pathol 2013;44:2690–5.
[34] Mannweiler S, Sygulla S, Winter E, et al. Two major pathways of penile carcinogenesis: HPV-induced penile cancers overexpress p16ink4a, HPV-negative cancers associated with dermatoses express p53, but lack p16ink4a overexpression. J Am Acad Dermatol 2013;69:73–81.
[35] Stankiewicz E, Prowse DM, Ng M, et al. Alternative HER/PTEN/Akt pathway activation in HPV positive and negative penile carcinomas. PLoS One 2011;6:e17517.
[36] Russo VC, Azar WJ, Yau SW, et al. IGFBP-2: the dark horse in metabolism and cancer. Cytokine Growth Factor Rev 2015;26:329–46.
[37] Yau SW, Azar WJ, Sabin MA, et al. IGFBP-2 – taking the lead in growth, metabolism and cancer. J Cell Commun Signal 2015;9:125–42.
[38] Lin Y, Jiang T, Zhou K, et al. Plasma IGFBP-2 levels predict clinical outcomes of patients with high-grade gliomas. Neuro Oncol 2009;11:468–76.
[39] Cohen P, Peehl DM, Stamey TA, et al. Elevated levels of insulin-like growth factor-binding protein-2 in the serum of prostate cancer patients. J Clin Endocrinol Metab 1993;76:1031–5.
[40] Kendrick ZW, Firpo MA, Repko RC, et al. Serum IGFBP2 and MSLN as diagnostic and prognostic biomarkers for pancreatic cancer. HPB (Oxford) 2014;16:670–6.
[41] Prowse DM, Ktori EN, Chandrasekaran D, et al. Human papillomavirus-associated increase in p16INK4A expression in penile lichen sclerosus and squamous cell carcinoma. Br J Dermatol 2008;158:261–5.
[42] Adimonye A, Stankiewicz E, Kudahetti S, et al. Analysis of the PI3K-AKT-mTOR pathway in penile cancer: evaluation of a therapeutically targetable pathway. Oncotarget 2018;9:16074–86.