Identification of prognostic and bone metastatic alternative splicing signatures in bladder cancer

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Research

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Abstract

Background ☐ Bladder cancer, originating from the epithelium of the urinary bladder, was the second most common malignancy in the urinary system with a high metastasis rate and poor post-metastasis prognosis. Alternative splicing events (ASEs) were regarded as important markers of tumor progression and prognosis, however, their roles in bladder cancer bone metastasis haven't been recognized.

Methods ☐ In order to explore the mechanism of ASEs in bladder cancer bone metastasis, we downloaded the RNA sequencing data and ASEs data of 412 samples of primary BLCAs from The Cancer Genome Atlas (TCGA) and TCGASpliceSeq databases. The Cox regression analysis was used to identify overall survival-related ASEs (OS-SEs), then, based on the OS-SEs screened by Lasso regression, we constructed the predict model. Finally, univariate and multivariate independent prognostic analysis were performed to prove it as an independent prognostic factor.

Results ☐ In this study, a predict model of OS in BLCA was constructed and the Area Under Curve of the model was 0.581. Its risk score was also proved to be an independent predictor with the good accuracy ($P < 0.001$). Among identified 390 SFs, Junction plakoglobin (JUP) was significantly correlated with overall survival and bone metastasis. In co-expression analysis, the co-expression pathway of ITGB4 was the glycosphingolipid biosynthesis ganglio series.

Conclusions ☐ We speculated that JUP regulating the ITGB4 might play a key role in bone metastasis of bladder cancer through the glycosphingolipid biosynthesis ganglio series pathway ($R = 0.220$, $P < 0.001$), which was also related to prognosis.

Background

Bladder cancer (BLCA), originating from the epithelium of the urinary bladder, is the fourth most common cancer in men and the second most common malignancy in the urinary system [1]. In the past few years, the incidence and mortality of bladder cancer have been rising gradually [2]. In 2018, there were 549,000 new cases of bladder cancer and 200,000 deaths worldwide [3]. There are two subtypes of bladder cancer, non-muscle invasive bladder cancer (NMIBC) and muscle invasive bladder cancer (MIBC). A large number of patients occurred bone metastasis at the terminal stage. By then, a total tumor resection is difficult. Even with cisplatin chemotherapy, bladder cancer patients with bone metastasis could not survive more than 14–15 months [4]. Thus, there is a pressing need to explore the mechanism of bone metastasis and predict the prognosis of patients with bladder cancer.

Alternative splicing (AS) is a pivotal determinant of genome complexity and an important mechanism for generating proteome diversity [5]. In the human genome, about 95% of the genes are alternatively spliced [6, 7], in turn, it also couples with the complexity of the genome. Thus, AS takes part in diverse mRNA isoforms spliced and protein variants translated. In this process, splicing factor (SF) works as regulatory catalyst of alternative splicing events (ASEs). The aberrant AS of some genes and somatic mutations of SFs were frequently found in tumors. They might influence the protein-protein interactions in cancer-

related pathways and modulate malignant transformation of cells, tumor cells invasion and metastasis [8]. Thus, identifying the dysregulated network of SFs and ASEs may provide the novel molecular biomarkers for prognosis, metastasis and therapy [9–12]. However, a comprehensive analysis of the prognostic value of ASEs in bladder cancer especially for bladder cancer bone metastasis is still lacking which aroused our interest.

In this study, RNA sequencing data and clinical information of BLCA patients were retrieved from TCGA database and ASE data were obtained from TCGASpliceSeq database [13]. Then univariate and multivariate Cox regression were performed to screen ASEs with prognostic value. Besides, a regulatory network of significantly co-expressed SFs and ASEs was built, and the co-expression relationship among Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and other ASEs with prognostic value and significant correlation with bone metastasis was detected. Interestingly, it provided reliable prognostic signals for BLCA patients.

Methods

Data extraction

The study was approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University (No. KEYAN-2018-LW-040). The bladder cancer RNA-seq data was downloaded from the cancer genome atlas (TCGA, <https://tcga-data.nci.nih.gov/tcga/>). The dataset included 412 samples of primary BLCAs, including 23 samples with bone metastasis and 389 samples without bone metastasis. Each tumor case was coincided with a patient with bladder cancer. The 412 samples were then matched with their corresponding entries in the TCGASpliceSeq database [13]. The ASE ID was consisted of the gene name, the ID number of the TCGASliceSeq database (AS ID) and alternative splicing type, for example, in the annotation term “STXBP2-47123-AP”, the STXBP2 was the gene name, 47123 was the AS ID and alternate promoter (AP) was the splicing pattern. Collection of clinicopathological data included gender, age, TNM staging, clinical stage, grade, survival status, and survival time.

Identification of OS-SEs and function enrichment analysis

Univariate Cox regression analysis was applied to identify splicing events associated with overall survival which were presented in 7 categories according to ASEs. The top 20 enrichments terms of Gene Ontology (GO) term and KEGG pathway of the genes in OS-SEs were taken into a further analysis.

Analysis of the prognostic values of the PIs

The top 20 OS-SEs with the lowest P value for each type of splicing pattern were selected for lasso regression and multivariate Cox regression. The variables that might lead to the over fitting of the model were removed and the OS-SEs with P values < 0.05 were selected to construct the prognostic index (PI, regression value of each sample in the multivariate Cox regression model). The PI was calculated using the following formula:

$$\beta_{OS-SE1} \times PS_{IOS-SE1} + \beta_{OS-SE2} \times PS_{IOS-SE2} + \dots + \beta_{OS-SEn} \times PS_{IOS-SEn}$$

Then, receiver operating characteristic curves (ROC) was used to discriminate and evaluate the efficacy of the PIs in predicting prognosis of BLCA. Meanwhile, the survival rate was calculated by Kaplan–Meier method and the significance of difference between survival curves of high-risk group and low-risk group was determined by log-rank test. Finally, taking PI obtained by Cox regression model as variable, univariate and multivariate independent prognostic analysis of OS-associated clinical features and PI were performed to prove it as an independent prognostic factor.

Construction of the regulatory network of ASE and SF in BLCA

A total of 390 SFs were acquired from the SpliceAid2 database (<http://www.introni.it/splicing.html>) [14]. Nonparametric tests were implemented to identify the relationships between splicing events which were corresponding to survival. Then Pearson correlation analysis was performed to screen the significantly correlated SFs and OS-SEs to construct a regulatory network in which the absolute value of cutoff correlation coefficient was 0.45 and the P value was 0.001.

Identification of KEGG pathways co-expressing with ASEs

Both ASEs in the regulatory network and bone metastasis-related were interleaved to determine, which were not only related to bone metastases, but also significantly associated with SFs as well as OS. Next, Gene Set Variation Analysis (GSVA) pathway analysis and univariate Cox regression analysis were performed to recognize the OS-related KEGG pathways. Lastly, KEGG pathways co-expressing with ASEs were identified to explore the potential mechanism of the bone metastasis of BLCA.

Multidimensional validation

To minimize bias, multiple databases including UALCAN [15], LinkedOmics [16], SurvExpress [17] and Gene Expression Profiling Interactive Analysis (GEPIA) [18] were applied to detect gene and protein expression levels of Co-expressed genes and key molecules in pathways at the tissue and cellular levels.

Statistical analysis

All statistical analyses were performed using R version 3.5.1 software (Institute for Statistics and Mathematics, Vienna, Austria; www.r-project.org) (Package: edgeR, ggplot2, rms, glmnet, preprocessCore, survminer, timeROC). For all statistical analyses, only two-sided $P < 0.05$ was examined statistically significant. UpSet plots were applied to visualize the associations between genes and the different types of SEs.

Results

Analysis of ASEs in BLCA

The overall design of this study was shown in Fig. 1. The ASEs in 412 BLCA and mRNA samples in the TCGA dataset were analyzed. Figure 2A displayed the number of genes involved in the entire ASEs, involving alternative splicing type, the number of each alternative splicing type and the corresponding number of genes. A total of 9,415 genes in the BLCA cohort were identified as ASEs: alternate acceptor (AA) events in 2,079 genes, alternate donor (AD) events in 1,814 genes, AP events in 2,924 genes, alternate terminator (AT) events in 3,465 genes, exon skip (ES) events in 5,879 genes, mutually exclusive exons (ME) events in 305 genes and retained intron (RI) events in 1,593 genes.

Identification of OS-SEs

The univariate Cox regression analysis was performed to identify the ASEs associated with OS of BLCA patient and presented as seven splicing event types. Figure 2A illustrated the number of ASEs in different types of splicing patterns and Fig. 2B showed the number of genes involved in OS-related ASEs, the number of splicing events types involved, the number of each splicing event type and the corresponding number of genes. Volcano plot (Fig. 3A) showed the distribution of ASEs unrelated to OS and those related to OS based on all SEs. As showed in the bubble charts of seven SEs (Fig. 3B-3H), the most relevant OS-SEs for each type were STRBP – 87504 – AA ($P < 0.001$), RTN4 – 53597 – AD ($P < 0.001$), KLF5 – 26049 – AP ($P < 0.001$), EVC2 – 68693 – AT ($P < 0.001$), DCTN5 – 35625 – ES ($P < 0.001$), TMEM104 – 217418 – ME ($P < 0.001$) and LCMT2 – 30228 – RI ($P < 0.001$), respectively.

Prognostic predictors of OS in BLCA

The top20 ASEs with the lowest P value were selected, and lasso regression was used for variable screening. According to the results, the three genes with the lowest cross-validation error were incorporated into the final Cox regression model as independent prognostic indicators of bladder cancer patients (Fig. 4A-4B). After that, the PI of each patient was calculated, and then BLCA patients were divided into low-risk group and high-risk group according to the median value of riskscore. Kaplan-Meier method was applied for survival analysis and significant difference was achieved between low-risk group and high-risk group ($P = 0.007$) (Fig. 4C). Besides, the calibration curve and receiver operating characteristic curve (ROC) displayed a good accuracy of the constructed predict model (area under the ROC curve, AUC: 0.713) (Fig. 4D). In addition, the risk curve, ranked by patient risk from low to high, changed with risk value and high risk would result in increased mortality (Fig. 4E-4F). Figure 4G revealed SUPT7L-53037-AD, DCTN5-35625-ES, TP73-327-ES, CUX1-81080-ES were low-risk ASEs.

Next, univariate and multivariate Cox regression analysis were performed to evaluate whether the constructed prognostic model was independent along with other clinical factors such as age, gender and tumor staging (Fig. 5). The results of both univariate (hazard ratio (HR, 95%CI): 1.026 (1.015–1.037), $P < 0.001$) and multivariate (HR, 95%CI: 1.020 (1.009–1.031), $P < 0.001$) Cox regression analysis were corrected by baseline information (gender, age, TNM stage, clinical stage, bone metastasis) showed that the PI generated from the multivariate model was an independent prognostic factor.

Correlation between OS-SEs and SF expression

Figure 6A displayed the regulatory network of significantly co-expressed SFs and AEs. Five SFs were significantly correlated with AEs. Among these, BCAS1 was correlated with 50 favorable OS-SEs (purple ellipses) negatively (green lines) and 24 adverse OS-SEs (red ellipses) positively (red lines); RNU4-1 was correlated with 58 favorable OS-SEs (purple ellipses) negatively (green lines) and 3 adverse OS-SEs (red ellipses) positively (red lines); JUP was correlated with 7 favorable OS-SEs (purple ellipses) negatively (green lines) and 7 adverse OS-SEs (red ellipses) positively (red lines). Then, the AEs both in the regulatory network and related to bone metastasis were considered as the intersections (Fig. 6B). SMOX-58619-AP (Fig. 6C, $P = 0.015$), INO80C-45170-AP (Fig. 6D, $P = 0.022$) and ITGB4-43489-ES (Fig. 6E, $P = 0.048$) were found to be significantly related to both bone metastasis and OS in the Venn plot.

Functional enrichment analysis

Co-expression analysis of OS-related KEGG pathways and AEs related to bone metastasis of bladder cancer, SFs and OS-related AEs was performed. As shown in Fig. 7, ITGB4 - 43489 - ES was highly co-expressed with the pathway of primary bile acid biosynthesis ($R = 0.170$), tryptophan metabolism ($R = 0.160$), glycerolipid metabolism ($R = 0.16$), glycosphingolipid biosynthesis ganglio series ($R = 0.220$) in the none-bone metastases group and bone metastases group but lowly co-expressed with the pathway of fructose and mannose metabolism ($R = -0.160$) and glycosylphosphatidylinositol gpi anchor biosynthesis ($R = -0.170$). SMOX - 58619 - AP was less expressed in pathways, Linoleic acid metabolism ($R = 0.180$) and Alpha linolenic acid metabolism ($R = 0.160$). Besides, INO80C - 45170 - AP was up-regulated in pyrimidine metabolism between the none-bone metastases group and the bone metastasis group. Through multidimensional validation, we speculated that JUP regulating the ITGB4 - 43489 - ES might play a key role in bone metastasis of bladder cancer through the glycosphingolipid biosynthesis ganglio series pathway, which was also related to prognosis.

Multidimensional validation

In order to detect gene and protein expression levels of key biomarkers the 7 OS-SEs, we conducted multidimensional validation using multiple databases (Table 1). Firstly, in pathway unification database, ST8SIA5, ST8SIA1, ST3GAL2, ST3GAL5 and B4GALNT1 were key molecules in glycosphingolipid biosynthesis ganglio series pathway. Then, in UALCAN database, we found JUP and ITGB4 were higher expressed in tumor while ST3GAL5 and ST8SIA1 were lower expressed in tumor. In the database of LinkedOmics, ST8SIA5 was related to OS and ST8SIA1 is related to staging. In the SurvExpress, ST8SIA5, ST3GAL5 and B4GALNT1 were associated with OS significantly. Meanwhile, the GEPIA database showed that JUP, ITGB4, ST3GAL2, ST3GAL5 and B4GALNT1 were higher expressed in tumor samples than in paired normal tissues. Finally, Fig. 8 summarizes the speculative mechanism diagram including JUP, ITGB4 - 43489 - ES and glycosphingolipid biosynthesis ganglio series pathway.

Table 1
Baseline information of 412 patients diagnosed with bladder cancer.

Variables	Total Patients(N = 412)
Age,years	
Mean $\pm$ SD	68.10 $\pm$ 10.57
Median(Range)	69 (34–90)
Gender	
Female	108 (26.21%)
Male	304 (73.79%)
Grade	
High Grade	388 (94.17%)
Low Grade	21 (5.10%)
unknow	3 (0.73%)
Stage	
Stage I	2 (0.485%)
Stage II	131 (31.80%)
Stage III	141 (34.22%)
Stage IV	136 (33.01%)
unknow	2 (0.485%)
T	
T0	1 (0.24%)
T1	3 (0.73%)
T2	38 (9.22%)
T2a	26 (6.31%)
T2b	56 (13.59%)
T3	43 (10.44%)
T3a	71 (17.23%)
T3b	82 (19.90%)
T4	11 (2.67%)

Variables	Total Patients(N = 412)
T4a	43 (19.44%)
T4b	5 (1.21%)
TX	1 (0.24%)
unknow	32 (7.77%)
M	
M0	196 (47.57%)
M1	11 (2.67%)
MX	202 (49.03%)
unknow	3 (0.73%)
N	
N0	239 (58.01%)
N1	47 (11.41%)
N2	76 (18.45%)
N3	8 (1.94%)
NX	36 (8.74%)
unknow	6 (1.46%)
Bone Metastasis	
Yes	23 (5.58%)
No	389 (94.42%)
Abbreviations:SD,Standard deviation; T,tumor; M,metastasis; N,regional lymph node.	

Table 2 The external validation of JUP, ITGB4, ST8SIA5, ST8SIA1, ST3GAL2, ST3GAL5 and B4GALNT1.

Database	JUP	ITGB4	ST8SIA5	ST8SIA1	ST3GAL2	ST3GAL5	B4GALNT1
UALCAN	Expression:P = 0.014	Expression:P < 0.001	Expression:P = 0.125	Expression:P < 0.001	Expression:P = 0.070	Expression:P = 0.036	Expression:P = 0.088
LinkedOmics	K-M analysis:P = 0.200	K-M analysis:P = 0.386	K-M analysis:P = 0.014	K-M analysis:P = 0.820	K-M analysis:P = 0.765	K-M analysis:P = 0.464	K-M analysis:P = 0.444
	M:P = 0.968	M:P = 0.589	M:P = 0.259	M:P = 0.898	M:P = 0.134	M:P = 0.505	M:P = 0.883
	Stage:P = 0.188	Stage:P = 0.588	Stage:P = 0.676	Stage:P < 0.001	Stage:P = 0.071	Stage:P = 0.405	Stage:P = 0.407
CCLE	upregulated in tumor	upregulated in tumor	downregulated in tumor	downregulated in tumor	upregulated in tumor	upregulated in tumor	upregulated in tumor
SurvExpress	K-M analysis:P = 0.633	K-M analysis:P = 0.760	K-M analysis:P = 0.044	K-M analysis:P = 0.112	K-M analysis:P = 0.061	K-M analysis:P = 0.013	K-M analysis:P = 0.006
GEPIA	upregulated in tumor	upregulated in tumor	no significant difference	downregulated in tumor	upregulated in tumor	upregulated in tumor	upregulated in tumor
The Human Protein Atlas	Tumor high	Tumor not detected	Tumor not detected	Tumor medium	Tumor medium	Tumor low	Tumor not detected
	Normal high	Normal medium	Normal not detected	Normal medium	Normal not detected	Normal not detected	Normal not detected

Discussion

Bladder cancer was the most common malignant tumor of the urinary system with the highest morbidity and mortality [19]. It had a high degree of malignancy and often presented invasive development. After surgery the risk of recurrence and metastasis was more than 45% in one year. Bone was a widespread metastatic site of solid tumors. Even with cisplatin chemotherapy, patients of bladder cancer with bone metastasis could survive less than 14–15 months [4]. Alternative splicing was considered as one of important biological processes during tumorigenesis and progression [8], particularly in the invasion and metastasis of tumor cells [9–11]. Alternative splicing events were key biomarkers for cancer diagnosis and treatment, as well as potential targets for drug discovery [20], however, few studies focused on the potential role of alternative splicing events in bone metastasis and prognosis of BLCA.

In this study, we firstly found that alternative splicing was associated with the occurrence and progression of bladder cancer, and had a certain relationship with the prognosis of bladder cancer. Three genes at the minimum cross-validation error point in Lasso regression were incorporated into the final Cox regression model as independent prognostic indicators affecting the prognosis of bladder cancer patients. ROC curve showed good evaluation results for the accuracy of the constructed predict model, and univariate and multivariate Cox regression analysis results proved that the prognostic model could be used as an independent prognostic factor. SMOX-58619-AP, INO80C-45170-AP and ITGB4-43489-ES were significantly related to the bone metastasis, splicing factor and survival. The three splicing events were co-expressed with the OS-related KEGG pathways, and after multiple databases, we speculated and constructed a final regulation model. For bladder cancer patients with bone metastasis, JUP could down-regulating the ITGB4 – 43489 – ES by the pathway of glycosphingolipid biosynthesis ganglio series which was also related to the prognosis.

Junction plakoglobin (JUP, γ -catenin), a member of the armadillo family of proteins [21], is a homolog of β -catenin and forms distinct complexes with cadherins and desmosomal cadherins, which is a key part of the extracellular matrix [22]. These catenin proteins mediated intercellular interactions and signal transduction between cells [23]. Since JUP is an adhesive protein, the lack of JUP expression can reduce cell-cell contact and increase its proliferation in the body and cancer cells [21]. In this study, by constructing the network of OS-SEs and prognosis-related SFs, we found JUP was one of the SFs that associated OS-SEs, OS and bone metastasis. Besides, a negative regulatory relationship was existed between JUP and ITGB4. Similar to our results, it was reported that JUP was a crucial SF affecting the metastasis and prognosis of other cancers. In oral squamous cell carcinoma, JUP promoted its proliferation, migration, invasion and was a potential prognostic marker [21]; In breast cancer, loss of JUP would trigger the decreasing contact between cells and the increasing the invasion and spread of breast cancer cells [24]; In addition, Syrigos et al. and Rieger et al. found that bladder cancer patients with an abnormal expression of JUP always had poor survival status, and the restoration of plakoglobin expression in bladder carcinoma cell lines could inhibit cell migration and tumorigenic potential [25, 26].

Integrin played a major role in signaling networks that promoted angiogenesis and tumor progression [27]. Genetic experiments suggested that tumor cells might be more dependent on specific integrin than normal cells and might be regulated by integrin signals at different stages of tumor progression [28]. Integrin Beta 4 (ITGB4) was the structural component that maintains the hemidesmosomes (HDs) of the epithelial architecture [29]. It was the laminin receptor in tumor cells and angiogenic endothelial cells [30]. Integrin beta4 was characterized by its 1017-amino acid long domain in the beta4 subunit which paired only with the $\alpha 6$ subunit, and the heterodimeric integrin $\alpha 6\beta 4$ played a role in the invasive and metastatic phenotype of various cancers [28, 31, 32]. Previous studies showed that ITGB4 was highly expressed in a variety of tumors [29, 33]. It participated in the proliferation, invasion and metastasis [34–36], and also associated with poor prognosis of some tumors [37, 38]. Leng et al. found that ITGB4 could enhance the tumor growth in hepatocellular carcinoma patients and promote lung metastasis by activation of FAK–AKT pathway [35]. In ovarian cancer, the Hh signaling pathway could induce cell migration and invasion through the activation of FAK, which was mediated by ITGB4 [39]. ITGB4 could also serve as a prognostic marker for breast cancer [38].

The up-regulation of ITGB4 in multiple cancer cells indicated that the redistribution of ITGB4 provided favorable conditions for cell proliferation and invasion [29]. In normal epithelial cells, ITGB4 bound to HDs and promoted the anchoring of epithelial cells to the basal membrane. But in cancer cells, ITGB4 was redistributed from HDs to the anterior edges of cells enriched in the lamellar and filamentous feet, enhancing tumor migration and invasion [40, 41]. In tumor tissues, phosphorylation of the cytoplasmic tail of ITGB4 led to its release from the semi-desmosome and its interaction with the growth factor receptor [42]. The phosphorylation of the cytoplasmic tail of ITGB4 released integrin $\alpha 6\beta 4$ from hemidesmosomes, which led to its interaction with growth factor receptors and the induction of growth signaling [42, 43]. Phosphoinositide 3-kinase (PI3K) and RhoA small gtpase were activated by integrin alpha 6 beta 4 bound to laminin. In addition, the interaction between integrin alpha6 beta4 and growth factor receptors included activation signaling pathways of the epidermal growth factor receptor family,

such as PI3K AKT, and MAPK signaling was involved in tumorigenesis and metastasis [44]. Therefore, similar to our hypothesis, ITGB4 was associated with bone metastasis of bladder cancer and could be used as a prognostic marker in bladder cancer.

To explore the regulation between JUP and ITGB4, the glycosphingolipid biosynthesis ganglio series pathway was identified as the co-expression signaling pathway through GSVA pathway analysis. Ganglioside (GS) was one kind of sugar sphingolipids containing sialic acid. It was the main component of animal cell membrane [45] and engaged in intercellular recognition, connection, movement and information transmission [46]. It was also associated with tumor differentiation and malignant transformation [47]. GM3, a single sialic acid containing ganglioside, regulated cell adhesion, growth and movement by altering the level of molecular tissue in the synaptic microzone of sugar genes and the activation of co-localization signaling molecules involved in cancer pathogenesis [48]. Previous studies had proved the significant accumulation of GM3 in non-muscle-invasive bladder cancer but a small quantity in muscle-invasive bladder cancer [45]. Furthermore, increased GM3 expression induced growth suppression of bladder cancer cells by brefeldin A [49].

However, there were inevitably some limitations in our study. First of all, the data used in this study was from the public source. Information on other confounding variables, such as smoking, was not available for analysis. But, given the large populations involved we would have anticipated that any differences in background factors would have been evenly distributed via randomization. Secondly, the samples were all from European, which might lead a selection bias. So, a multiple databases validation by could reduce this bias by examining the expression levels of co-expressed genes and key molecules in all the other sources we can found. Thirdly, our result was not validated by wet experiments. But we designed a more comprehensive dry experiment, with plenty of statistical methods, calculation and multiple databases validations. By now, it was the first report to discover that ASEs were involved in GSVA pathway in bone metastases in bladder cancer patients. ASEs were firstly used in the prediction of prognosis in bladder cancer patients. Therefore, our findings could have a nice guiding role for clinicians to make a reasonable prediction for bone metastases for bladder cancer patients.

Conclusion

In this study, we speculated that JUP regulating ITGB4 might play a key role in bone metastasis of bladder cancer through the pathway glycosphingolipid biosynthesis ganglio series, which was also related to prognosis. Based on the comprehensive bioinformatics analysis, a predict model for forecasting the prognosis of BLCA patients was constructed, and its reliability was demonstrated by its high AUC value. The identified alternative splicing events were significantly correlated with bone metastasis and had certain prognostic value for bladder cancer patients.

Abbreviations

ASEs

alternative splicing events
BLCA
bladder urothelial carcinoma;
TCGA
The Cancer Genome Atlas
OS
overall survival
SE
splicing events
SF
splicing factor
AS
alternative splicing
KEGG
Kyoto Encyclopedia of Genes and Genomes
GO
Gene Ontology
PI
prognostic index
ROC
receiver operating characteristic curve
GSVA
Gene Set Variation Analysis
GEPIA
Gene Expression Profiling Interactive Analysis
AA
alternate acceptor
AD
alternate donor
AP
alternate promoter
AT
alternate terminator
ES
exon skip
ME
mutually exclusive exons
RI
retained intron
AUC

area under the ROC curve

TNM stage

T:tumor, N:regional lymph node, M:metastasis

ORF

open reading frame

HDs

hemidesmosomes.

JUP

Junction plakoglobin

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Tongji University School of Medicine

Consent for publication

No individual person's data in any form were involved in this study.

Availability of data and materials

All data generated or analyzed during this study are included in this published article. For additional information, please contact the author.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

All authors read and approved the final manuscript.

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References

1.
Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. *CA Cancer J Clin.* 2015;65:5–29.
2.
Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, Jemal A, Yu XQ, He J. Cancer statistics in China, 2015. *CA Cancer J Clin.* 2016;66:115–32.
3.
WANG Ning LS, Lei YANG, Xi ZHANG, Yan-nan YUAN. LI Hui-chao, JI Jia-fu Interpretation on the report of Global Cancer Statistics 2018. *Journal of Multidisciplinary Cancer Management(Electronic Version)* 2019.01–10.
4.
von der Maase H, Sengelov L, Roberts JT, Ricci S, Dogliotti L, Oliver T, Moore MJ, Zimmermann A, Arning M. Long-term survival results of a randomized trial comparing gemcitabine plus cisplatin, with methotrexate, vinblastine, doxorubicin, plus cisplatin in patients with bladder cancer. *J Clin Oncol.* 2005;23:4602–8.
5.
Liu Y, Gonzalez-Porta M, Santos S, Brazma A, Marioni JC, Aebersold R, Venkitaraman AR, Wickramasinghe VO. Impact of Alternative Splicing on the Human Proteome. *Cell Rep.* 2017;20:1229–41.
6.
Pan Q, Shai O, Lee LJ, Frey BJ, Blencowe BJ. Deep surveying of alternative splicing complexity in the human transcriptome by high-throughput sequencing. *Nat Genet.* 2008;40:1413–5.
7.
Wang ET, Sandberg R, Luo S, Khrebtkova I, Zhang L, Mayr C, Kingsmore SF, Schroth GP, Burge CB. Alternative isoform regulation in human tissue transcriptomes. *Nature.* 2008;456:470–6.
8.
Climente-Gonzalez H, Porta-Pardo E, Godzik A, Eyras E. The Functional Impact of Alternative Splicing in Cancer. *Cell Rep.* 2017;20:2215–26.
9.
Aversa R, Sorrentino A, Esposito R, Ambrosio MR, Amato A, Zambelli A, Ciccodicola A, D'Apice L, Costa V. Alternative Splicing in Adhesion- and Motility-Related Genes in Breast Cancer. *Int J Mol Sci* 2016, 17.
10.
Uhlen M, Fagerberg L, Hallstrom BM, Lindskog C, Oksvold P, Mardinoglu A, Sivertsson A, Kampf C, Sjostedt E, Asplund A, et al. Proteomics. Tissue-based map of the human proteome. *Science.* 2015;347:1260419.
11.
He RQ, Zhou XG, Yi QY, Deng CW, Gao JM, Chen G, Wang QY. Prognostic Signature of Alternative Splicing Events in Bladder Urothelial Carcinoma Based on Spliceseq Data from 317 Cases. *Cell Physiol Biochem.* 2018;48:1355–68.
12.
Marzese DM, Manughian-Peter AO, Orozco JIJ, Hoon DSB. Alternative splicing and cancer metastasis: prognostic and therapeutic applications. *Clin Exp Metastasis.* 2018;35:393–402.

13.

Ryan M, Wong WC, Brown R, Akbani R, Su X, Broom B, Melott J, Weinstein J. TCGASpliceSeq a compendium of alternative mRNA splicing in cancer. *Nucleic Acids Res.* 2016;44:D1018–22.

14.

Piva F, Giulietti M, Nocchi L, Principato G. SpliceAid: a database of experimental RNA target motifs bound by splicing proteins in humans. *Bioinformatics.* 2009;25:1211–3.

15.

Chandrashekar DS, Bashel B, Balasubramanya SAH, Creighton CJ, Ponce-Rodriguez I, Chakravarthi B, Varambally S. UALCAN: A Portal for Facilitating Tumor Subgroup Gene Expression and Survival Analyses. *Neoplasia.* 2017;19:649–58.

16.

Vasaikar SV, Straub P, Wang J, Zhang B. LinkedOmics: analyzing multi-omics data within and across 32 cancer types. *Nucleic Acids Res.* 2018;46:D956-d963.

17.

Aguirre-Gamboa R, Gomez-Rueda H, Martinez-Ledesma E, Martinez-Torteya A, Chacolla-Huaringa R, Rodriguez-Barrientos A, Tamez-Pena JG, Trevino V. SurvExpress: an online biomarker validation tool and database for cancer gene expression data using survival analysis. *PLoS One.* 2013;8:e74250.

18.

Tang Z, Li C, Kang B, Gao G, Li C, Zhang Z. GEPIA: a web server for cancer and normal gene expression profiling and interactive analyses. *Nucleic Acids Res.* 2017;45:W98-w102.

19.

Baras AS, Drake C, Liu JJ, Gandhi N, Kates M, Hoque MO, Meeker A, Hahn N, Taube JM, Schoenberg MP, et al. The ratio of CD8 to Treg tumor-infiltrating lymphocytes is associated with response to cisplatin-based neoadjuvant chemotherapy in patients with muscle invasive urothelial carcinoma of the bladder. *Oncoimmunology.* 2016;5:e1134412.

20.

Le KQ, Prabhakar BS, Hong WJ, Li LC. Alternative splicing as a biomarker and potential target for drug discovery. *Acta Pharmacol Sin.* 2015;36:1212–8.

21.

Fang J, Xiao L, Zhang Q, Peng Y, Wang Z, Liu Y. Junction plakoglobin, a potential prognostic marker of oral squamous cell carcinoma, promotes proliferation, migration and invasion. *J Oral Pathol Med* 2019.

22.

Lewalle JM, Bajou K, Desreux J, Mareel M, Dejana E, Noel A, Foidart JM. Alteration of interendothelial adherens junctions following tumor cell-endothelial cell interaction in vitro. *Exp Cell Res.* 1997;237:347–56.

23.

Cowin P, Kapprell HP, Franke WW, Tamkun J, Hynes RO. Plakoglobin: a protein common to different kinds of intercellular adhering junctions. *Cell.* 1986;46:1063–73.

24.

- Holen I, Whitworth J, Nutter F, Evans A, Brown HK, Lefley DV, Barbaric I, Jones M, Ottewell PD. Loss of plakoglobin promotes decreased cell-cell contact, increased invasion, and breast cancer cell dissemination in vivo. *Breast Cancer Res.* 2012;14:R86.
- 25.
- Syrigos KN, Harrington K, Waxman J, Krausz T, Pignatelli M. Altered gamma-catenin expression correlates with poor survival in patients with bladder cancer. *J Urol.* 1998;160:1889–93.
- 26.
- Rieger-Christ KM, Ng L, Hanley RS, Durrani O, Ma H, Yee AS, Libertino JA, Summerhayes IC. Restoration of plakoglobin expression in bladder carcinoma cell lines suppresses cell migration and tumorigenic potential. *Br J Cancer.* 2005;92:2153–9.
- 27.
- Hood JD, Cheresch DA. Role of integrins in cell invasion and migration. *Nat Rev Cancer.* 2002;2:91–100.
- 28.
- Guo W, Giancotti FG. Integrin signalling during tumour progression. *Nat Rev Mol Cell Biol.* 2004;5:816–26.
- 29.
- Liu SY, Ge D, Chen LN, Zhao J, Su L, Zhang SL, Miao JY, Zhao BX. A small molecule induces integrin beta4 nuclear translocation and apoptosis selectively in cancer cells with high expression of integrin beta4. *Oncotarget.* 2016;7:16282–96.
- 30.
- Giancotti FG. Targeting integrin beta4 for cancer and anti-angiogenic therapy. *Trends Pharmacol Sci.* 2007;28:506–11.
- 31.
- Giancotti FG, Tarone G. Positional control of cell fate through joint integrin/receptor protein kinase signaling. *Annu Rev Cell Dev Biol.* 2003;19:173–206.
- 32.
- Hynes RO. Integrins: bidirectional, allosteric signaling machines. *Cell.* 2002;110:673–87.
- 33.
- Kettunen E, Nicholson AG, Nagy B, Wikman H, Seppanen JK, Stjernvall T, Ollikainen T, Kinnula V, Nordling S, Hollmen J, et al. L1CAM, INP10, P-cadherin, tPA and ITGB4 over-expression in malignant pleural mesotheliomas revealed by combined use of cDNA and tissue microarray. *Carcinogenesis.* 2005;26:17–25.
- 34.
- Yang ZY, Jiang H, Qu Y, Wei M, Yan M, Zhu ZG, Liu BY, Chen GQ, Wu YL, Gu QL. Metalloproteinase-1 regulates invasion and migration of gastric cancer cells partially through integrin beta4. *Carcinogenesis.* 2013;34:2851–60.
- 35.
- Leng C, Zhang ZG, Chen WX, Luo HP, Song J, Dong W, Zhu XR, Chen XP, Liang HF, Zhang BX. An integrin beta4-EGFR unit promotes hepatocellular carcinoma lung metastases by enhancing anchorage independence through activation of FAK-AKT pathway. *Cancer Lett.* 2016;376:188–96.
- 36.

- Ferraro A, Kontos CK, Boni T, Bantounas I, Siakouli D, Kosmidou V, Vlassi M, Spyridakis Y, Tsipras I, Zografos G, Pintzas A. Epigenetic regulation of miR-21 in colorectal cancer: ITGB4 as a novel miR-21 target and a three-gene network (miR-21-ITGBeta4-PDCD4) as predictor of metastatic tumor potential. *Epigenetics*. 2014;9:129–41.
- 37.
- Grossman HB, Lee C, Bromberg J, Liebert M. Expression of the alpha6beta4 integrin provides prognostic information in bladder cancer. *Oncol Rep*. 2000;7:13–6.
- 38.
- Brendle A, Lei H, Brandt A, Johansson R, Enquist K, Henriksson R, Hemminki K, Lenner P, Forsti A. Polymorphisms in predicted microRNA-binding sites in integrin genes and breast cancer: ITGB4 as prognostic marker. *Carcinogenesis*. 2008;29:1394–9.
- 39.
- Chen Q, Xu R, Zeng C, Lu Q, Huang D, Shi C, Zhang W, Deng L, Yan R, Rao H, et al. Down-regulation of Gli transcription factor leads to the inhibition of migration and invasion of ovarian cancer cells via integrin beta4-mediated FAK signaling. *PLoS One*. 2014;9:e88386.
- 40.
- Kashyap T, Germain E, Roche M, Lyle S, Rabinovitz I. Role of beta4 integrin phosphorylation in human invasive squamous cell carcinoma: regulation of hemidesmosome stability modulates cell migration. *Lab Invest*. 2011;91:1414–26.
- 41.
- Gagnoux-Palacios L, Dans M, van't Hof W, Mariotti A, Pepe A, Meneguzzi G, Resh MD, Giancotti FG. Compartmentalization of integrin alpha6beta4 signaling in lipid rafts. *J Cell Biol*. 2003;162:1189–96.
- 42.
- Stewart RL, O'Connor KL. Clinical significance of the integrin alpha6beta4 in human malignancies. *Lab Invest*. 2015;95:976–86.
- 43.
- Shaw LM, Rabinovitz I, Wang HH, Toker A, Mercurio AM. Activation of phosphoinositide 3-OH kinase by the alpha6beta4 integrin promotes carcinoma invasion. *Cell*. 1997;91:949–60.
- 44.
- Li XL, Liu L, Li DD, He YP, Guo LH, Sun LP, Liu LN, Xu HX, Zhang XP. Integrin beta4 promotes cell invasion and epithelial-mesenchymal transition through the modulation of Slug expression in hepatocellular carcinoma. *Sci Rep*. 2017;7:40464.
- 45.
- Kawamura S, Ohyama C, Watanabe R, Satoh M, Saito S, Hoshi S, Gasa S, Orikasa S. Glycolipid composition in bladder tumor: a crucial role of GM3 ganglioside in tumor invasion. *Int J Cancer*. 2001;94:343–7.
- 46.
- Hakomori S. Structure, organization, and function of glycosphingolipids in membrane. *Curr Opin Hematol*. 2003;10:16–24.
- 47.

Wang H, Isaji T, Satoh M, Li D, Arai Y, Gu J. Antitumor effects of exogenous ganglioside GM3 on bladder cancer in an orthotopic cancer model. *Urology*. 2013;81:210 e211–5.

48.

Hakomori SI, Handa K. GM3 and cancer. *Glycoconj J*. 2015;32:1–8.

49.

Satoh M, Ito A, Nojiri H, Handa K, Numahata K, Ohyama C, Saito S, Hoshi S, Hakomori SI. Enhanced GM3 expression, associated with decreased invasiveness, is induced by brefeldin A in bladder cancer cells. *Int J Oncol*. 2001;19:723–31.

Figures

Figure 2

Figure 3

The UpSet plot of SEs and OS-SEs. (A) The number of AEs in different types of splicing patterns; (B) The number of OS-SEs in different types of splicing patterns. Abbreviation: AA, alternate acceptor; AD, alternate donor; AP, alternate promoter; AT, alternate terminator; ES, exon skip; ME, mutually exclusive exons; RI, retained intron.

Figure 5

Enrichment analysis of ASEs and bubble charts showing top 20 OS-SEs in seven types of splicing patterns. (A) The volcano plot displaying the prognosis-related and no significant ASEs, respectively. (B) Bubble chart of AA. (C) Bubble chart of AD. (D) Bubble chart of AP. (E) Bubble chart of AT. (F) Bubble chart of ES. (G) Bubble chart of ME. (H) Bubble chart of RI. Abbreviation: AA, alternate acceptor; AD, alternate donor; AP, alternate promoter; AT, alternate terminator; ES, exon skip; ME, mutually exclusive exons; RI, retained intron.

Figure 8

Establishment and assessment of the predict model. (A-B) Lasso regression for OS-SEs screening and removing high correlation genes to prevent over-fitting of the model. (C) Kaplan-Meier survival curves for patients in the low and high subgroups of the predict model demonstrating that risk score could significantly forecast the prognosis of patients with BLCA. (D) ROC curves demonstrating the accuracy of the model (AUC: 0.713). (E) The risk curve of each sample ranking by risk from low to high. (F) The

scatter plot showing the trend of change in risk value and the increase in patient mortality as the risk increased and illustrating the clinical status with green and red dots representing survival and death, respectively. (G) The heatmap of expression level of 4 OS-SEs filtered by Lasso regression. Abbreviation: AA, alternate acceptor; AD, alternate donor; AP, alternate promoter; AT, alternate terminator; ES, exon skip; ME, mutually exclusive exons; RI, retained intron.

Figure 10

Cox regression analysis for evaluating the independent prognostic value of the risk score. (A) univariate and (B) multivariate Cox regression analysis verify that risk score can be the independent prognostic factor of BLCA.

Figure 11

Alternative splicing network and clinical relevance. (A) Regulatory network of significantly co-expressed alternative splicing factors and alternative splicing events. The shape of arrow represents the splicing

factor, the red circle shows high risk alternative splicing and the purple circle shows low risk alternative splicing. The red and green lines represent the positive and negative regulatory relationships between AS and SF respectively. (B) Venn plot OS-SEs related to clinical status and bone metastasis. (C) Beeswarm plots displaying SMOX-58619-AP significantly related to bone metastasis. (D) Beeswarm plots displaying INO80C-45170-AP significantly related to bone metastasis. (E) Beeswarm plots displaying ITGB4-43489-ES significantly related to bone metastasis.

Figure 14

CorHeatmap of KEGG pathways and alternative splicing events that had prognostic value and were significantly associated with bone metastasis. GSEA pathway analysis and univariate Cox regression analysis identified survival related KEGG pathways, and co-expressed alternative splicing events related to prognosis and bone metastasis with survival related KEGG pathways.

Figure 16

The speculative mechanism diagram including JUP, ITGB4-43489-ES and glycosphingolipid biosynthesis ganglio series pathway.

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