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The splicing factor PTBP1 promotes expression of oncogenic splice variants and predicts poor prognosis in patients with non-muscle invasive bladder cancer

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Translational relevance

PTBP1 expression positively correlates with NMIBC progression and with worse clinical outcome of patients. Mechanistically, PTBP1 regulates pro-survival features and modulates alternative splicing of bladder cancer-related genes in NMIBC cell lines and patient specimens. Thus, PTBP1 expression and its splicing signature can represent novel outcome-predictor markers for NMIBC.

Abstract

Purpose: Non-muscle invasive bladder cancer (NMIBC) is a malignant disease characterized by high heterogeneity, which corresponds to dysregulated gene expression and alternative splicing (AS) profiles. Bioinformatics analyses of splicing factors potentially linked to bladder cancer progression identified the heterogeneous nuclear ribonucleoprotein I (i.e. PTBP1) as candidate. This study aimed at investigating whether PTBP1 expression associates with clinical outcome in NMIBC patients.

Experimental Design: A cohort of 152 patients presenting primary NMIBC (pTa-pT1) was enrolled. Primary NMIBCs were assessed for PTBP1 expression by immunohistochemistry (IHC) and the results were correlated with clinical data using Kaplan–Meier curves and Cox regression analyses. Cell proliferation and survival assays were performed to assess the function of PTBP1. Furthermore, the impact of PTBP1 on the AS pattern of specific bladder cancer-related genes was investigated in cancer cell lines and in patient’s specimens.

Results: Public datasets querying highlighted a positive correlation between PTBP1 expression and NMIBC progression, which was then confirmed by IHC analysis. High PTBP1 expression was associated with worse clinical outcome in terms of incidence of tumor relapse and survival in NMIBC patients. Interestingly, down-regulation of PTBP1 in bladder cancer cell lines affected pro-survival features. Accordingly, PTBP1 modulated AS of bladder cancer-related genes in cell lines and patient’s specimens.

Conclusion(s): PTBP1 expression correlates with disease progression, poor prognosis and worse survival in NMIBC patients. Down-regulation of PTBP1 expression affects pro-survival features of bladder cancer cells and modulates AS of genes with relevance for bladder cancer, suggesting its role as outcome-predictor in this disease.

Introduction

Bladder cancer is the sixth most common cancer in men, with an estimated 429,800 cases diagnosed in 2012 and 165,100 deaths for the disease worldwide (1). It is typically subdivided in three main categories on the basis of management goals and prognosis: non-muscle invasive bladder cancer (NMIBC), muscle invasive bladder cancer and metastatic bladder cancer. NMIBC represents the most frequent form and includes carcinoma in situ (stage Tis), papillary lesions confined to the urothelium (stage Ta) or invading the lamina propria (stage T1) (2). Complete trans-urethral resection of bladder tumors (TURBT) is the routine initial diagnostic and therapeutic step in management. However, over 50% NMIBCs will recur and up to 25% will progress to muscle-invasive disease (3). Due to probability of recurrence and progression, NMIBC requires repeated surveillance and intervention and is among the most expensive cancers to treat from diagnosis to death (4).

Criteria assessing patient and tumor characteristics provide valuable information about disease recurrence, progression and proposed treatments. The European Organisation for Research and Treatment of Cancer (EORTC) electronic risk calculator (<http://www.eortc.be/tools/bladdercalculator>) is commonly used to assess recurrence and progression potential of newly diagnosed cancers. The parameters used are tumor size and number, pathological stage and grade, presence of carcinoma in situ (CIS), and prior recurrence rate (5). Ease of use and absence of expensive molecular tests represent the main advantages of this and other scoring models (6,7). However, reproducibility of pathologic stage and grade is modest and remains a critical clinical concern.

Strong efforts are currently aimed at identifying molecular markers with robust diagnostic and prognostic value for NMIBC (8). Although several molecular markers are currently approved by the U.S. Food and Drug Administration (FDA) and its European counterpart (9), their value in predicting NMIBC recurrence and progression is limited and none of them is recommended by clinical guidelines (10). Thus, identification of valuable markers or therapeutic targets that reduce the likelihood of recurrence and/or progression is a clinical priority for NMIBC management.

Dysregulation of splicing is emerging as a key feature of human cancers with therapeutic perspective (11). Splicing leads to excision of introns and ligation of exons from the precursor mRNA (pre-mRNA) and is orchestrated by the spliceosome, a macromolecular machinery composed of five small nuclear ribonucleoprotein particles and hundreds of auxiliary proteins (12). When the exon-intron boundaries (splice sites) display high levels of conservation, exons are almost always included in the mRNA (constitutive exons), whereas

exons lacking strong consensus sequences are subjected to regulation (alternative exons). In this case, exon recognition is tuned by *trans*-acting splicing factors (SFs) that bind to *cis*-regulatory sequences. The interplay between antagonistic SFs determines whether a target exon is included or skipped through a process named alternative splicing (AS) (12). Since most splice isoforms engage specific interactions and behave as distinct proteins (13), AS increases the coding potential of genomes and represents an evolutionary advantage (14). However, its flexible regulation is prone to errors and defective splicing contributes to neoplastic transformation (11,13). In this regard, splicing inhibition is envisioned as an effective anti-cancer therapy, as many tumors are very sensitive to this approach (11).

The main classes of SFs are the serine-arginine (SR) proteins and the heterogeneous nuclear ribonucleoproteins (hnRNPs), which often act antagonistically in AS regulation (12). Members of both classes, like SRSF1 (15,16) and hnRNPA1, A2 and I (also known as PTBP1) (17,18), were shown to play oncogenic functions. Notably, although AS dysregulation has been reported also in bladder cancer (19), limited information is available regarding the SFs responsible for this process and their possible association with prognosis. Herein, we have performed bioinformatics search for SR and hnRNP proteins associated with bladder cancer progression. Our study identified hnRNPI/PTBP1 as a factor linked to disease progression and suggest PTBP1 as a new prognostic factor and possible therapeutic target for NMIBC progression.

Materials and Methods

Bioinformatics analysis

R2 genomics platform (<http://r2.amc.nl>) and OncoPrint database (www.oncoPrint.org) were used to evaluate the association of splicing factors with bladder cancer grade using Hoglund, Dyrskjot and Sanchez-Carbayo datasets (20-22).

Study population and ethics statement

Patients with histologically proven primary pTa and pT1 bladder urothelial carcinoma were enrolled in the study. Inclusion criteria were: age ≥ 18 years, adequate bone-marrow reserve, normal renal and liver function and Eastern Cooperative Oncology Group performance status between 0 and 2 (23). Exclusion criteria were: previous bladder cancer; non-urothelial carcinomas; previous or concomitant urinary-tract carcinoma in situ and urothelial carcinoma of the upper urinary tract and urethra; bladder capacity less than 200 mL; untreated urinary tract infection; severe systemic infection; urethral strictures preventing endoscopic catheterization; previous radiotherapy to the pelvis; other concurrent chemotherapy, radiotherapy, treatment with biological response modifiers; other malignant diseases within 5 years of trial registration (except for adequately treated basal-cell or squamous-cell skin cancer, and in situ cervical cancer); pregnancy; other factors precluding study participation.

All patients underwent TURBT of endoscopically detected tumors, ensuring that muscle was included in resected samples, as specified by European Association of Urology guidelines (2). Before TURBT, a cold-cup biopsy of apparently non-tumor-bearing and tumor were taken and stored at -80°C until analysis. Tumors were staged in accordance with the TNM classification (24) and biopsies were graded according to WHO classifications (25). Risk categories for recurrence and progression were assessed in accordance with EORTC risk tables for NMIBC (5). Within 6 hours of TURBT, patients received a single intravesical instillation of 40 mg mitomycin. 4-6 weeks after TURBT, patients without muscle in resected samples, positive or suspect cytology, carcinoma in situ, stage T1, or grade 3 tumors underwent restaging transurethral resection, random cold-cup bladder and prostatic urethra biopsies, and upper-urinary-tract imaging. No adjuvant intravesical therapy was given to patients with low-risk NMIBC. Patients with intermediate-risk and high-risk NMIBC were scheduled to receive adjuvant long-term intravesical chemotherapy with mitomycin, or immunotherapy with bacillus Calmette-Guerin, starting ~ 3 weeks after TURBT procedures. Patients were followed-up with abdominal ultrasonography, urinary cytology and cystoscopy every 3 months for 2 years, twice during the third year, and yearly thereafter.

All procedures performed in studies involving human participants were in accordance with the ethical standards and with the Declaration of Helsinki. The institutional review board approved the study design and all enrolled patients signed an informed consent form providing details of treatments.

Immunohistochemistry and statistical analysis

PTBP1 immunohistochemistry (IHC) was performed on formalin-fixed, paraffin-embedded tissues obtained from 152 primary pTa-pT1 NMIBC patients. Antigen was retrieved by microwave treatment at 750 W for 10 min in 10 mM sodium citrate buffer (pH 6.0). Sections were incubated for 60 min at room temperature with 1:500 anti-PTBP1 antibody (sc-16547, Santa Cruz Biotechnology, Santa Cruz, CA), or non-immune serum as control, using the LSAB signal amplification kit (K0690, Dako, Glostrup, Denmark). PTBP1 expression was defined by presence of nuclear staining in tumor cells. IHC analysis was done in blind by two pathologists (M.P., R.L.) unaware of clinic-pathologic information, with 80% concordance in evaluation. 30 cases (19.7%) in which evaluation of PTBP1 expression differed by >10% between the two observers were re-evaluated. After consensus was achieved, the absolute inter-observer variability (mean difference% \pm SD) recorded was 3.95% \pm 3.14.

The relationship between PTBP1 expression and clinic-pathological parameters were investigated by Pearson's χ^2 test or Fisher's exact test. All clinical analyses were performed on an intent to treat basis. Primary endpoint was disease-free interval (DFS), defined as time from initial TURBT randomisation to first cystoscopy noting recurrence. Secondary endpoints were progression free (PFS), overall (OS), and disease specific survival (DSS). Starting from initial TURBT: PFS was defined as time until onset of muscle invasive disease as recorded by pathological assessment of transurethral-resection samples or biopsy samples; OS as time until death from any cause; DSS as time until death from bladder cancer. Patients without recurrence or progression were censored at the last cystoscopy, and those lost to follow-up were censored at the last known day of survival. Endpoints were studied by the Kaplan-Meier method and comparisons were made by the log-rank test. Association of PTBP1 expression with outcome, adjusted for other prognostic factors, was tested by Cox's proportional hazards model. Appropriateness of the proportional hazard assumption was assessed by plotting the log cumulative hazard functions over time and checking for parallelism. SPSS Version 15.0 (SPSS, Chicago, IL) was used throughout and $P < 0.05$ was considered statistically significant.

Cell cultures and manipulations

Bladder cancer cell lines, RT4, RT112 and EJ (26), were purchased and kindly provided by Dr. Francesca Velotti (La Tuscia University, Viterbo, Italy), and further authentication has not

been performed. Bladder cancer cell lines were cultured in RPMI 1640 medium (LONZA) supplemented with 10% FBS (Gibco) at 37°C with 5% CO₂. Mycoplasma contamination of cell cultures was routinely tested by Hoechst 33258 stain (27), every two-three weeks. Upon thawing, bladder cancer cell lines have been subcultured for not more than 15 passages. RNAi experiments, protein extractions and immunofluorescence analyses were performed as previously described (28,29).

For clonogenic, cell cycle, proliferation and adhesion assays, cells were silenced twice with CTRL or PTBP1 siRNAi (80nM). Colony-forming assay was performed as described (30). Briefly, 24 hrs after transfection, 400 cells were plated in 60mm dishes and cultured for one week. After fixation-staining for 30min with glutaraldehyde 6.0% (vol/vol)/crystal violet 0.5% (wt/vol) solution, colonies with $n > 50$ cells were counted.

For cell cycle/subG1 analyses, cells were pulse-labeled with 10 μ M BrdU for 45min before harvesting and fixed with a 30% phosphate-buffered saline/70% ethanol solution for 30 minutes in ice. Hypodiploid events and cell cycle were evaluated by flow cytometry using propidium iodide (PI) staining (20 μ g/ml) and anti-BrdU antibody in the presence of 13 Kunitz units/ml ribonuclease A as described (31). 15×10^3 events were counted with FacsCalibur flow cytometer (Becton Dickinson) and analyzed using Flow-Jo program (Becton Dickinson).

Cell death was evaluated by staining for Annexin V (1 μ g/ml) and PI (1 μ g/ml) (eBioscience) and analyzed by flow cytometry (FACSCanto; BD Biosciences). The combination of Annexin V and PI staining discriminates live cells (Annexin V/PI double negative) from apoptotic cells (Annexin V/PI double positive).

Splicing assay, UV-crosslinked RNA immunoprecipitation (CLIP) and qRT-PCR analyses

RNA was extracted from cells using Trizol reagent (Invitrogen) and PCR analyses were performed as described (28,29). Primers are listed in Supplementary Information. Quantitative expression level of CD44 variable exons was calculated by Δ Ct method relative to total CD44. CLIP assays were performed as extensively described and RNA associated with PTBP1 was represented as percentage of input (32).

Results

PTBP1 expression correlates with poor prognosis in tumor bladder patients.

Public datasets (R2 genomics analysis and visualization platform, <http://r2.amc.nl>) were queried for correlation between expression of SR proteins and hnRNPs and bladder cancer progression. Expression of PTBP1 (i.e. hnRNPI) was found to significantly correlate with disease progression in the Hoglund cohort including 308 patients (Fig. 1A; Supplementary Fig. S1A). PTBP1 expression also resulted significantly higher in invasive T1 stage compared to non-invasive Ta stage cancers (Fig. 1B). Analysis of two other datasets (<https://www.oncomine.org>: Dyrskjot and Sanchez-Carbayo datasets) confirmed that PTBP1 expression is increased in infiltrating and superficial bladder cancers compared to normal tissue (Supplementary Fig. S1B-D). More importantly, Kaplan-Meier analysis highlighted significant association between high PTBP1 mRNA expression and low OS probability ($p < 0.001$; Fig. 1C). By contrast, expression of other SFs was either unchanged or slightly affected, or associated only with G3 tumors (i.e. hnRNPA2B1, hnRNPC, hnRNPL, SRSF7 and SRSF10; Fig. 1A; Supplementary Fig. S1A).

To determine whether the bioinformatics analysis of mRNA expression levels in public databases represents a valuable tool to identify proteins with oncogenic potential, PTBP1 up-regulation was also tested at the protein level by Western blot analysis using a cohort of surgical tumor (T) and non-tumor (NT) specimens from 50 NMIBC patients. PTBP1 protein was significantly increased in the neoplastic lesions compared to the adjacent non-neoplastic urothelium ($p = 0.003$; Fig. 2A). To extend this analysis, we enrolled a cohort of 178 patients. After initial TURBT, we excluded 13 patients because of concomitant urothelial carcinoma of the upper urinary tract ($n = 3$) and prostatic urethra ($n = 4$), histology other than pure urothelial carcinoma ($n = 4$), and consent refusal ($n = 2$). Restaging TURBT was performed in 69 patients (54 with high-risk disease and 15 with multifocal intermediate-risk disease) and histological findings showed that 10 patients with residual stage pTa or pT1 tumor were eligible, whereas 9 with concomitant carcinoma in situ (pTis) and 4 with muscle invasive disease (stage pT2) were ineligible. Of the remaining 152 patients (see clinical features and tumor characteristics in Table 1), seventy-four cases (48.7%) were non-invasive papillary carcinoma (pTa) and seventy-eight (51.3%) were tumors invading sub-epithelial connective tissue (pT1). According with EORTC risk tables classification for NMIBC (5), the cancer progression risk was low in 27 cases (17.7%), intermediate in 70 cases (46.1%) and high in 54 cases (36.2%; Table 1).

Percentage of PTBP1-positive cells was significantly higher in tumor specimens with respect to the non-neoplastic urothelium (Supplementary Fig. S2A), and it ranged from 3 to 98% (mean \pm SE=40.7 \pm 2.9) in tumor tissues. Examples of PTBP1 expression in patients with low-, intermediate-, and high-risk of cancer progression are shown in Fig. 2B. Notably, significant increase in PTBP1 expression was associated with risk of cancer progression (Fig. 2C).

To dichotomize PTBP1 expression, a cut-off of 6% positive cells was chosen according to the receiver operating characteristic (ROC) curve analysis (AUC=0.779, $p < 0.001$; Supplementary Fig. S2B,C). Tumors with $>6\%$ positive cells ($n=105$) were considered PTBP1^{High} and those with $\leq 6\%$ positive cells ($n=47$) were PTBP1^{Low}. PTBP1 expression directly correlated with pT1 tumors ($p < 0.001$), grade 3 tumors ($p < 0.001$), and tumors with high risk of progression ($p < 0.001$) (Table 1). We also found direct correlation between PTBP1^{High} and the number of patients experiencing tumor recurrence ($p=0.001$), cancer progression ($p=0.021$), overall mortality ($p=0.047$), and cancer mortality ($p=0.029$) (Table 1). Furthermore, Kaplan-Meier analysis indicated that PTBP1^{High} expression was significantly associated with lower DFS, PFS, OS, and DSS rates (Fig. 2D). In particular, survival rates for patients with PTBP1^{High} and PTBP1^{Low} tumors were 39.0% vs. 68.1% (DFS; $p < 0.001$), 89.5% vs. 100.0% (PFS; $p=0.023$), 57.1% vs. 74.5% (OS; $p=0.006$), and 90.5% vs. 100.0% (DSS; $p=0.024$). Lastly, univariate analysis showed that PTBP1^{High} was significantly associated with poor DFS (hazard ratio [HR], 2.6; 95% confidence interval [CI], 1.5-4.6; $p=0.001$), and OS (HR, 2.4; 95% CI, 1.3-4.5; $p=0.008$) (Supplementary Table S1). Multivariate analysis demonstrated that the only factors negatively impacting on survival were tumor grade (DFS; $p=0.018$), and age of patients at diagnosis (OS; $p < 0.001$) (Supplementary Table S2).

These observations confirm the predictive value of our bioinformatics analysis of deposited mRNA expression levels and highlight PTBP1 as a novel marker of poor prognosis and disease progression, thus suggesting its oncogenic role in bladder cancer.

PTBP1 depletion affects pro-survival features of bladder cancer cells.

To investigate whether PTBP1 was functionally relevant for bladder cancer cells, we knocked down its expression by two rounds of transfection with siRNAs (siPTBP1) and obtained an almost complete depletion in three bladder cancer cell lines (RT4, RT112 and EJ; Supplementary Fig. S3A). Silencing of PTBP1 expression significantly reduced growth of all cell lines as measured by clonogenic assays (Fig. 3A; Supplementary Fig. S3B). Cytofluorimetric analysis of the cell cycle indicated that PTBP1 knockdown resulted in the reduction of cells engaged in active S phase (BrdU-positive cells) and in the concomitant

increase of cells in inactive S phase (not incorporating BrdU but stalled with DNA content between 2N and 4N) (Fig. 3B; Supplementary Fig. S3C). Moreover, depletion of PTBP1 dramatically increased the population of cells with a sub-G1 DNA content (Fig. 3C; Supplementary Fig. S3D), suggesting that stalling in S phase was followed by cell death. Indeed, double staining with Annexin V and PI (Fig. 3D; Supplementary Fig. S3E) and immunofluorescence analysis of caspase-3 cleavage (Supplementary Fig. S3F) confirmed the significant increase in apoptotic cells after depletion of PTBP1 in all three cell lines.

To test whether depletion of PTBP1 affects the response of bladder cancer cells to chemotherapeutic treatment with mitomycin C, we used a suboptimal concentration of the drug (0.03 μ M) and set out conditions to reduce PTBP1 without strongly affecting cell survival (lower siRNA concentration and single round of transfection). Under these conditions, silencing of PTBP1 enhanced the cytotoxic effect of mitomycin C on bladder cancer cells death, as indicated by double staining with Annexin V and PI (Fig. 3E; Supplementary Fig. S3G) and analysis of caspase 3 cleavage (Supplementary Fig. S3H).

These results demonstrate that PTBP1 favors bladder cancer cell proliferation and survival and that it protects them from chemotherapeutic treatment.

PTBP1 regulates bladder cancer-relevant splice variants

PTBP1 is best known for its role in AS (33) and expression of oncogenic splice variants has been positively associated with disease progression in bladder cancer patients (19,34). We noted that some of these AS events are potential PTBP1 targets (Supplementary Table S3) (35,36). To test whether PTBP1 promotes the expression of these pro-oncogenic variants in bladder cancer cells, we examined its impact on the splicing pattern of eight genes. The oncogenic splice variants of seven of these genes were expressed in all three cell lines, whereas *CD44* alternative variants were detected only in RT112 cells (Supplementary Fig. S4A,B). Notably, knockdown of PTBP1 reverted splicing of the pro-oncogenic variant of all its potential bladder cancer-related target genes (Fig. 4A,B) (35,36). On the other hand, exon 3 in *PIK4CB* and exon 5-6 in *LRRFIP2*, which are not its predicted target exons (Supplementary Table S3), were unaffected by PTBP1 depletion.

In addition to being involved in cell proliferation (*PKM* and *NUMB*) or cell death (*FAS*), which are affected by PTBP1 depletion (Fig. 3), several of the PTBP1 target genes encode for proteins that regulate the cell cytoskeleton and adhesion (*ACTN1*, *MACF1*, *TPM1*, *CD44* and *CTNND1*). Accordingly, we found that knockdown of PTBP1 impaired cell adhesion in bladder cancer cell lines (Supplementary Fig. S4C), indicating that changes in the splicing

pattern of these genes may be functionally relevant. In this regard, *CD44* is a prototypic example of AS-regulated gene. It contains nineteen exons, nine of which (V2-V10) are alternative spliced to yield multiple variants (CD44v) that encode for variable extracellular ligand-binding domains of this trans-membrane glycoprotein and whose inclusion correlates with tumor progression and metastasis (37). To investigate which of the variable CD44 exons is regulated by PTBP1, we performed semi-quantitative PCR (sqPCR) analyses in RT112 cells. Interestingly, inclusion of variable exons between v2 and v7 was reduced in cells depleted of PTBP1, whereas that of other variable exons (v8 to v10) was not (Supplementary Fig. S5A). Quantitative PCR (qPCR) confirmed this exon-specific regulation in PTBP1-depleted cell (Supplementary Fig. S5B).

PTBP1 directly binds in proximity of regulated exons in bladder cancer cells

To test whether PTBP1 plays a direct role in the regulation of its target exons, we investigated PTBP1 recruitment on pre-mRNAs *in vivo* by performing UV-crosslink and RNA immunoprecipitation (CLIP) assays (32). PTBP1 exerts a position-dependent effect on AS by promoting exon inclusion when it binds in the downstream intron and exon skipping when it binds in the upstream intron (Supplementary Fig. S5C,D upper panels) (35,36). Thus, CLIP experiments were performed in presence of RNase I (fragment size ~200bp) (32) and we analyzed PTBP1 recruitment near the downstream intron of the positively regulated exon v7 of *CD44* and exon 99 of *MACF1* (Supplementary Fig. S5C) and the upstream intron of the negatively regulated exon 6a of *TPM1* and exon 2 of *CTNND1* (Supplementary Fig. S5D). In both cases, we detected a significant enrichment of PTBP1 binding in the target regions with respect to non regulated exons (Supplementary Fig. S5C,D). These results demonstrate that PTBP1 specifically binds target exons *in vivo* and highlight its direct role in AS regulation of genes with strong relevance for bladder cancer.

PTBP1 expression associates with CD44 splicing regulation in bladder cancer patients

To evaluate whether regulation of splicing by PTBP1 was also detected in NMIBC specimens, we performed qPCR analysis of CD44 variable exons that are regulated (v5 and v7) or not (v9) by PTBP1. We selected 18 patients with variable PTBP1 levels (0-95% positive cells in IHC) from the cohort (Fig. 5A). Strikingly, PTBP1 expression was positively correlated with inclusion of its target exons v5 and v7, whereas the PTBP1-insensitive v9 exon was not significantly associated (Fig. 5B). These results strongly indicate that PTBP1 expression influences splicing outcome in bladder cancer patients.

Discussion

NMIBC mortality rate and management costs are strikingly high, underlining the need for valuable prognostic markers and therapeutic targets. In this regard, the splicing signature of human cancers is recently emerging as a sophisticated marker to distinguish tumor subtypes and precisely stratify patients (38). Herein, an unbiased approach to identify SFs with prognostic value in NMIBC undertaken by querying public databases has highlighted PTBP1 as a key predictive factor. We report that PTBP1 expression levels positively associate with disease progression and worse prognosis in patients. Accordingly, PTBP1 promotes pro-survival features of bladder cancer cells and favors splicing of oncogenic variants in both cell lines and patients. Thus, our study shows the predictive value of bioinformatics analysis of deposited gene expression datasets. Furthermore, it uncovers an oncogenic role for PTBP1 in NMIBC and identifies new potential prognostic and therapeutic targets for this disease.

Aberrant splicing regulation often confers selective advantage to tumor cells by favoring oncogenic splice variants of cancer-related genes (13,39,40). For instance, up-regulation of PTBP1 in human cancer cells affected glycolytic metabolism by promoting splicing of the PKM2 variant (17), leading to acquisition of chemotherapy resistance in pancreatic cancer (18). Likewise, we found that knockdown of PTBP1 in bladder cancer cells augmented the cytotoxic effects of mitomycin C. More importantly, PTBP1 expression level exhibits a strong prognostic value in bladder cancer, as we found a significant correlation between PTBP1 expression, disease progression and worse outcome in a relatively large cohort of patients. Indeed, although, multivariate analysis did not identify PTBP1 expression as an independent predictor of outcome, its expression level is included among the variables that impact on disease progression and outcome, while Kaplan-Meier curves revealed that high expression of PTBP1 correlates with poor DFS, PFS, OS and DSS. These observations reveal that PTBP1 can be considered a valuable marker to stratify NMIBC patients in term of risk prediction and evaluation of its expression levels could support the current histological classification and improve clinical evaluation of patients. This information may also help guiding clinical decisions regarding the follow-up, such as choosing between conservative adjuvant therapy or more aggressive treatment strategies.

We observed a 2 to 3-fold increase of PTBP1 expression in bladder cancer through public datasets analysis. Although this change in expression might appear limited, comparable small changes in expression of other splicing factors were previously shown to trigger oncogenic transformation, like in the case of SRSF1 (15,16). On the contrary, strong up-regulation of

splicing factors can be toxic for the cell (15,41). Thus, the reported increase in expression of PTBP1 in bladder cancer might be sufficient for its oncogenic function. Nevertheless, we cannot rule out the possibility that post-transcriptional regulation of PTBP1 also contributes to its oncogenic potential. For instance, alternative usage of exon 9 generates PTBP1 isoforms showing different target specificity and splicing activity (42). This regulation might impact on the oncogenic role of PTBP1 in bladder cancer and might also influence the multivariate analysis. However, since antibodies specific for these PTBP1 isoforms are not available and no data regarding isoform-specific expression was deposited in the public datasets, we could not investigate this aspect in our cohort of patients.

Expression of pro-oncogenic splice variants is associated with bladder cancer progression (19). We noted that several of these bladder-cancer regulated genes were potential target of PTBP1. Accordingly, knockdown of PTBP1 expression readily reverted splicing of these genes to the less oncogenic variant. Moreover, we report a positive correlation between the expression level of PTBP1 and the inclusion of variable exons v5 and v7 of *CD44* in NMIBC patient's specimens. Based on these observations, we hypothesize that the relationship between AS changes in these genes and bladder cancer progression might rely on PTBP1 expression. PTBP1 likely exerts a direct effect on these targets, as we detected its recruitment in proximity of the regulated exons in bladder cancer cells. Notably, PTBP1-regulated genes encode for proteins related to cell survival (*FAS*), proliferation (*NUMB*, *PKM*), cytoskeleton organization (*ACTN1*, *MACF1*, *TPM1* and *CTNND1*) and interaction with the extracellular matrix (*CD44*). Furthermore, depletion of PTBP1 affected proliferation, survival and adhesion of bladder cancer cells, suggesting a causative role for this SF in oncogenic features of NMIBC cells. For instance, the increase in apoptosis observed in absence of PTBP1 may rely on a splicing switch in favor of the pro-apoptotic and membrane-associated *FAS* variant (+E6). By contrast, repression of exon 6 inclusion by PTBP1 generates a soluble pro-survival isoform of *FAS* (s*FAS*) (43). Likewise, PTBP1 promotes inclusion of variable exon v6 in *CD44* and this isoform promotes stemness and metastasis in colorectal cancer cells (44). Increased inclusion of *CD44* variable exons has been reported in bladder cancer (34,45,46). Our study now correlates inclusion of variable exons v5 and v7 with PTBP1 expression in 19 NMIBC patients, suggesting that deregulation of *CD44* AS in bladder cancer may be directly caused by altered expression of PTBP1. Noteworthy, although we focused on PTBP1 splicing targets with direct relevance for bladder cancer, our findings do not rule out possible effects of PTBP1 on other variants of the same or other genes in this disease.

In conclusion, our study shows that up-regulation of PTBP1 in bladder cancer cells alters their transcriptome in favor of pro-oncogenic splice variants and correlates with worse outcome in patients. Thus, we suggest that PTBP1 expression and its splicing signature represent novel outcome-predictor markers for NMIBC.

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Figure legends

Figure 1. mRNA expression level of the splicing factor PTBP1 is increased in bladder tumor. Analysis of the expression of splicing factors in tumor bladder patients at stage G1 versus stage G2 and stage G3 (A) and at stage Ta (non-invasive papillary carcinoma) versus stage T1 (invasive carcinoma) (B). Boxes indicate the median (horizontal line); Whiskers, distances from the largest and smallest value to each end of the box; Dots, outliers. Mean values were compared with the two-tailed unpaired t-test. Data used for the analysis are from GSE 32894 Tumor Bladder-Hoglund-dataset (308 patients) deposited in R2 genomics analysis and visualization platform (<http://r2.amc.nl>). ns=no significance; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$. C) Kaplan-Meier overall survival analysis of the total Hoglund cohort based on the cut-off value of PTBP1 expression levels calculated by the R2 system. The difference between the curves for PTBP1 high and PTBP1 low groups were compared by log-rank test.

Figure 2. PTBP1 expression correlates with bladder cancer patient outcome. A) Representative western-blot analysis of PTBP1 expression in tumor (T) and adjacent non-tumor (NT) surgical specimens from 4 of the 50 NMIBC patients examined (upper panel). Tubulin was used as loading control. Densitometric analysis of PTBP1 expression relative to tubulin in non-tumor and tumor specimens is shown in the dots graph below ($n=50$; ** $p < 0.001$, independent-sample t-test). B) Representative images of IHC analysis of PTBP1 expression in two patients with low- (1,2), intermediate- (3,4), and high- (5,6) risk of progression. C) Expression of PTBP1 in NMIBC patients according to risk of cancer progression. The expression of PTBP1 was assessed by IHC analysis of paraffin-embedded sections (Mean percent of positive tumor cells \pm Standard Error; ** $p < 0.001$, independent-sample t-test). D) Kaplan-Meier disease free survival (DFS), progression free survival (PFS), overall survival (OS), and disease specific survival (DSS) analyses of 152 NMIBC patients stratified in high (solid gray line) and low (dashed black line) PTBP1 expression levels in tumor tissue. Statistical analysis was performed by the Long-rank test.

Figure 3. Modulation of PTBP1 expression affects pro-survival features in bladder cancer cells. A) Representative images of clonogenic assay performed with RT4 bladder cancer cells transfected twice with CTRL (siCTRL) or PTBP1 (siPTBP1) siRNAs. Bar graph represents the percentage of colonies formation with respect to siCTRL cells [set as 100%; mean \pm standard deviation (SD) of three independent experiments]. B,C) Representative

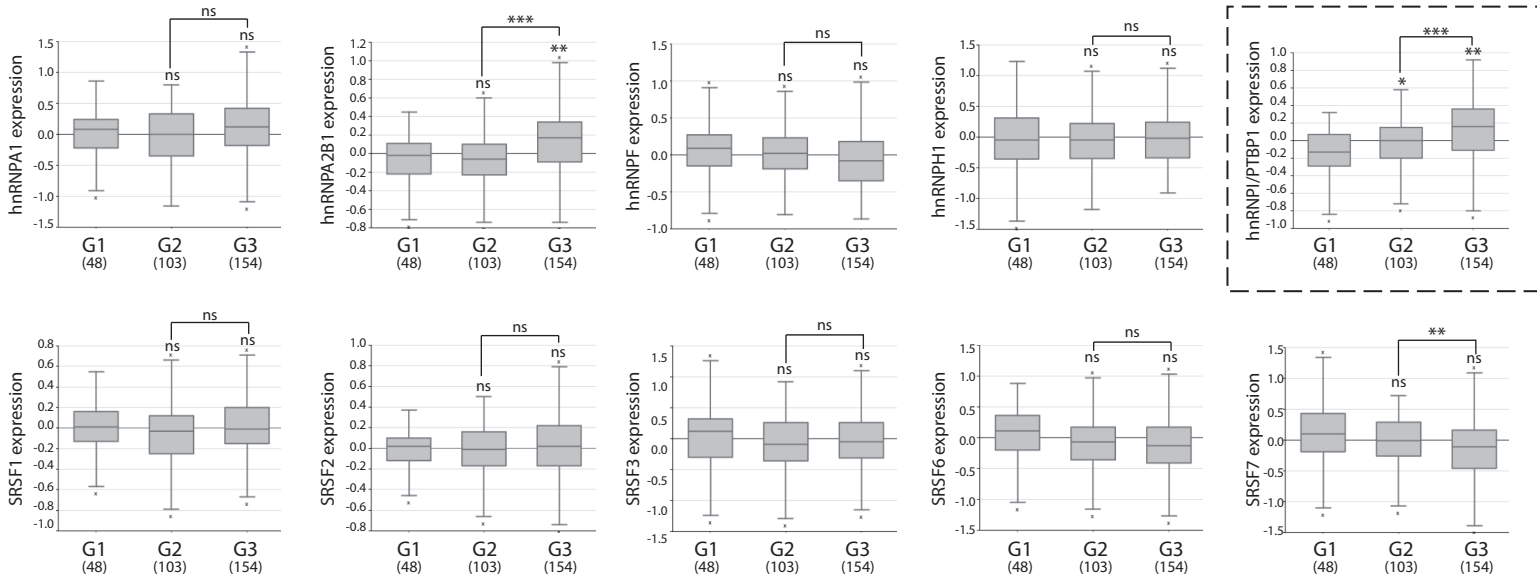
bivariate plot profiles of cytometric analysis showing DNA content (propidium iodide, PI) versus bromodeoxyuridine (BrdU) incorporation (B) and plot profiles of PI incorporation (C). RT4 cells were pulse labeled with BrdU for 45 min and successively stained with BrdU antibody and PI. G1, S, G2 and inactive S (S inac) phase gates (square boxes) and the percentage of cells at each phase are indicated (B). Bar graphs show the percentage of cells in S and inactive S (B) and sub-G1 (C) phases. D,E) Representative plot profiles of Annexin V/PI cytometric analysis of RT4 cells transfected twice (D) or once (E) with the indicated siRNAs and treated (E) or not (D) with suboptimal amount of mitomycin C (0.03 μ M) for 24hrs. Bar graphs represent the percentage of double positive Annexin V and PI cells (mean \pm SD of three independent experiments). Statistical analyses were performed by the paired Student's t-test (A-D) or One way Anova (E) (* $P \leq 0.05$, ** $P \leq 0.01$).

Figure 4. PTBP1 modulates alternative splicing of bladder cancer-related gene. A) sqPCR of *in vivo* splicing assays performed in bladder cancer cell lines silenced with control (-) or PTBP1 (+) siRNAs of specific bladder-cancer gene targets. Schematic representation of alternative splicing events (left panel) analyzed is shown on the left. Exons (boxes), introns (lines) are indicated (left panel). The oncogenic splice variant is indicated (*) to the right of the agarose gel. B) Densitometric analysis (right graphs) of the splicing assays shown in (A) (mean \pm SD, n=3). Statistical analyses were performed by the paired Student's t-test (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$; ns: not significant).

Figure 5. Inclusion of CD44 variable exons v5 and v7 correlates with PTBP1 expression in bladder cancer patients. A) PTBP1 expression assessed by immunohistochemistry analysis is expressed as percentage of positive cells. B) Pearson correlations between PTBP1 and variable exons V5, V7 and V9 expression, assessed by qPCR analysis, in 18 NMIBC patients.

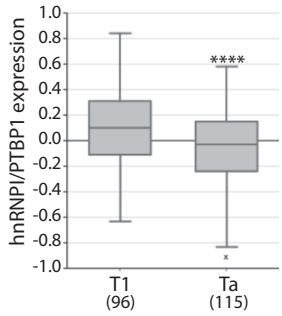
A

Tumor Bladder-Hougland



B

Tumor Bladder-Hougland



C

Tumor Bladder-Hougland

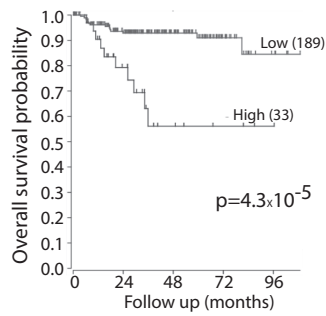


Figure 1

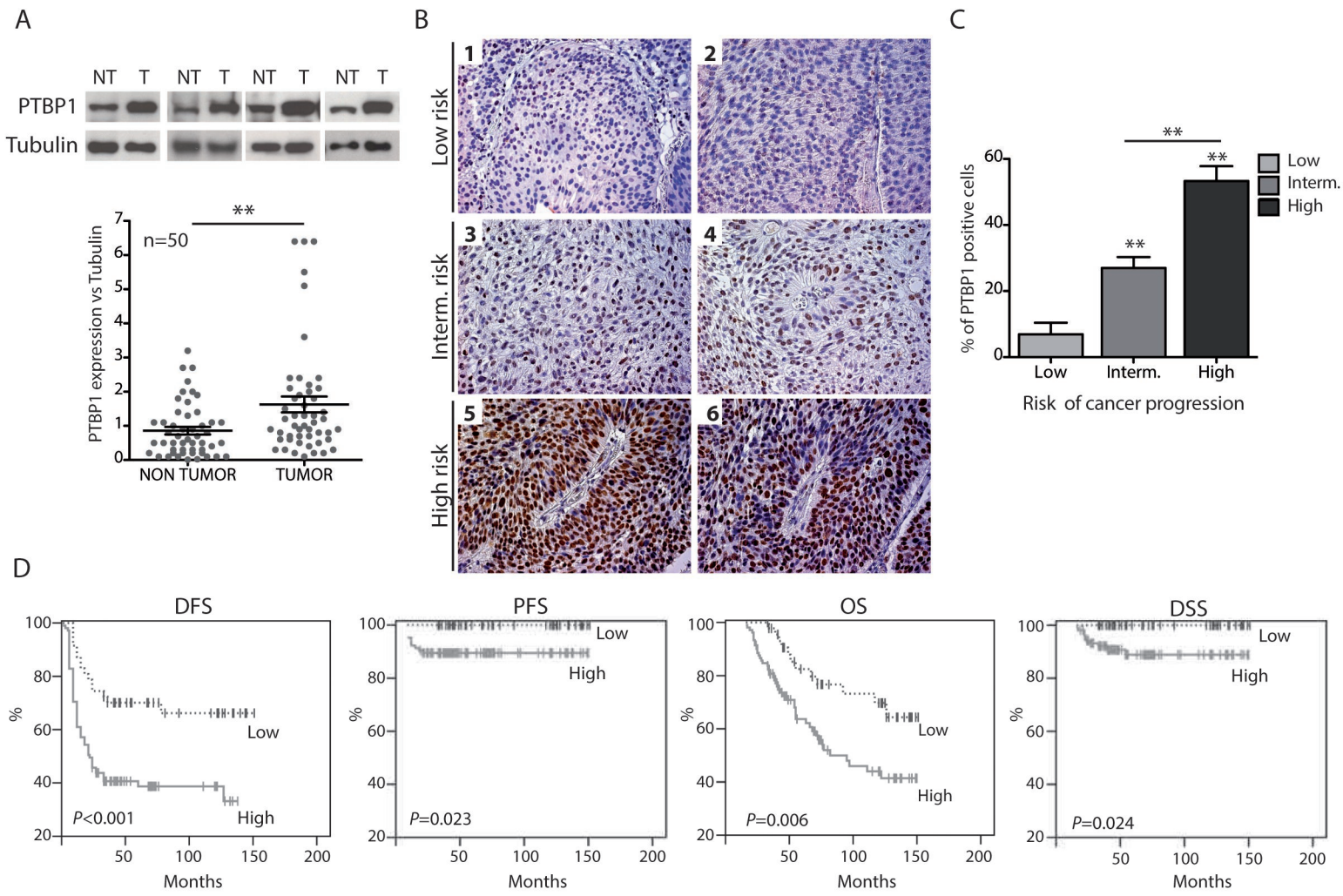
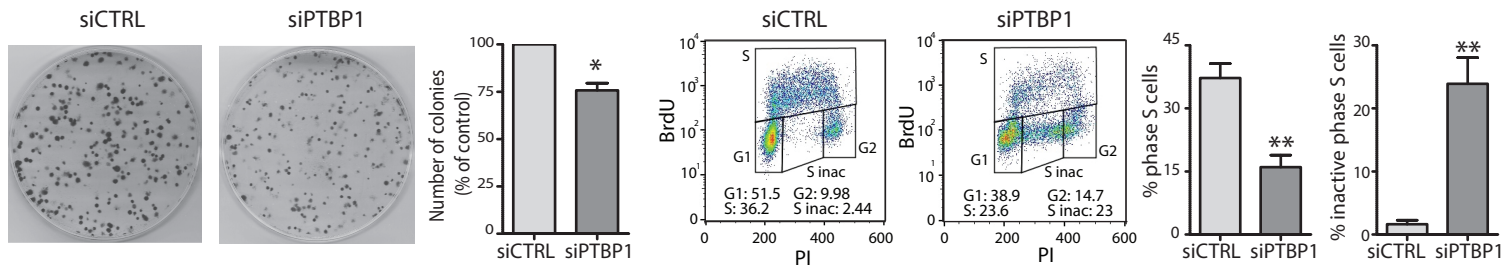


Table 1. PTBP1 status according to clinical and pathological features and to clinical outcome of patients (n = 152)

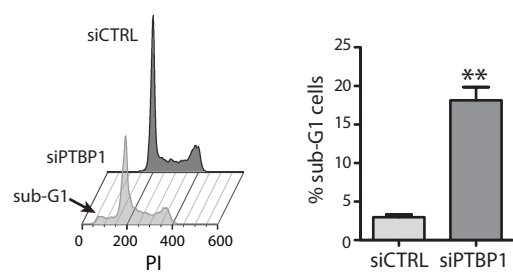
Variable	PTBP1		<i>P</i> ^o
	Low: n (%)	High: n (%)	
Age			
≤ 65	17 (36.2)	33 (31.4)	0.580
> 65	30 (63.8)	72 (68.6)	
Gender			
Male	36 (76.6)	84 (80.0)	0.670
Female	11 (23.4)	21 (20.0)	
pT classification			
pTa	42 (89.4)	32 (30.5)	<0.001*
pT1	5 (10.6)	73 (69.5)	
Tumor Grade			
1-2	43 (91.5)	55 (52.4)	<0.001*
3	4 (8.5)	50 (47.6)	
Risk of cancer progression			
Low	21 (44.7)	6 (5.7)	<0.001*
Intermediate	22 (46.8)	48 (45.7)	
High	4 (8.5)	51 (48.6)	
Recurrence			
No	32 (68.1)	41 (39.0)	0.001*
Yes	15 (31.9)	64 (61.0)	
Progression			
No	47 (100.0)	94 (89.5)	0.021*
Yes	0 (0.0)	11 (10.5)	
Overall Survival			
Death free	35 (74.5)	60 (57.1)	0.047*
Death from any cause	12 (25.5)	45 (42.9)	
Disease specific survival			
Death free	47 (100.0)	95 (90.5)	0.029*
Death from bladder cancer	0 (0.0)	10 (9.5)	

^oPearson's χ^2 test; *Statistically significant

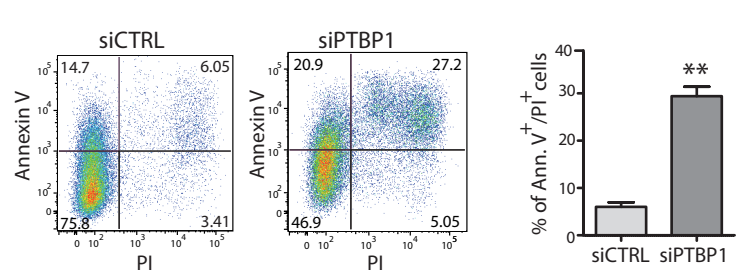
A



C



D



E

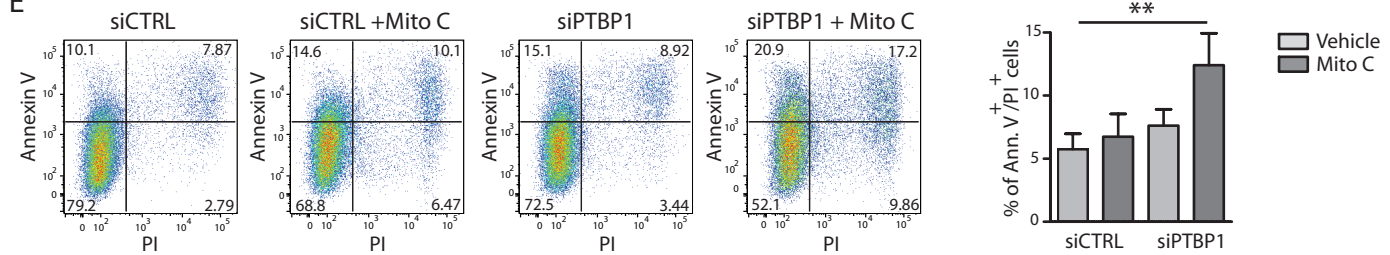
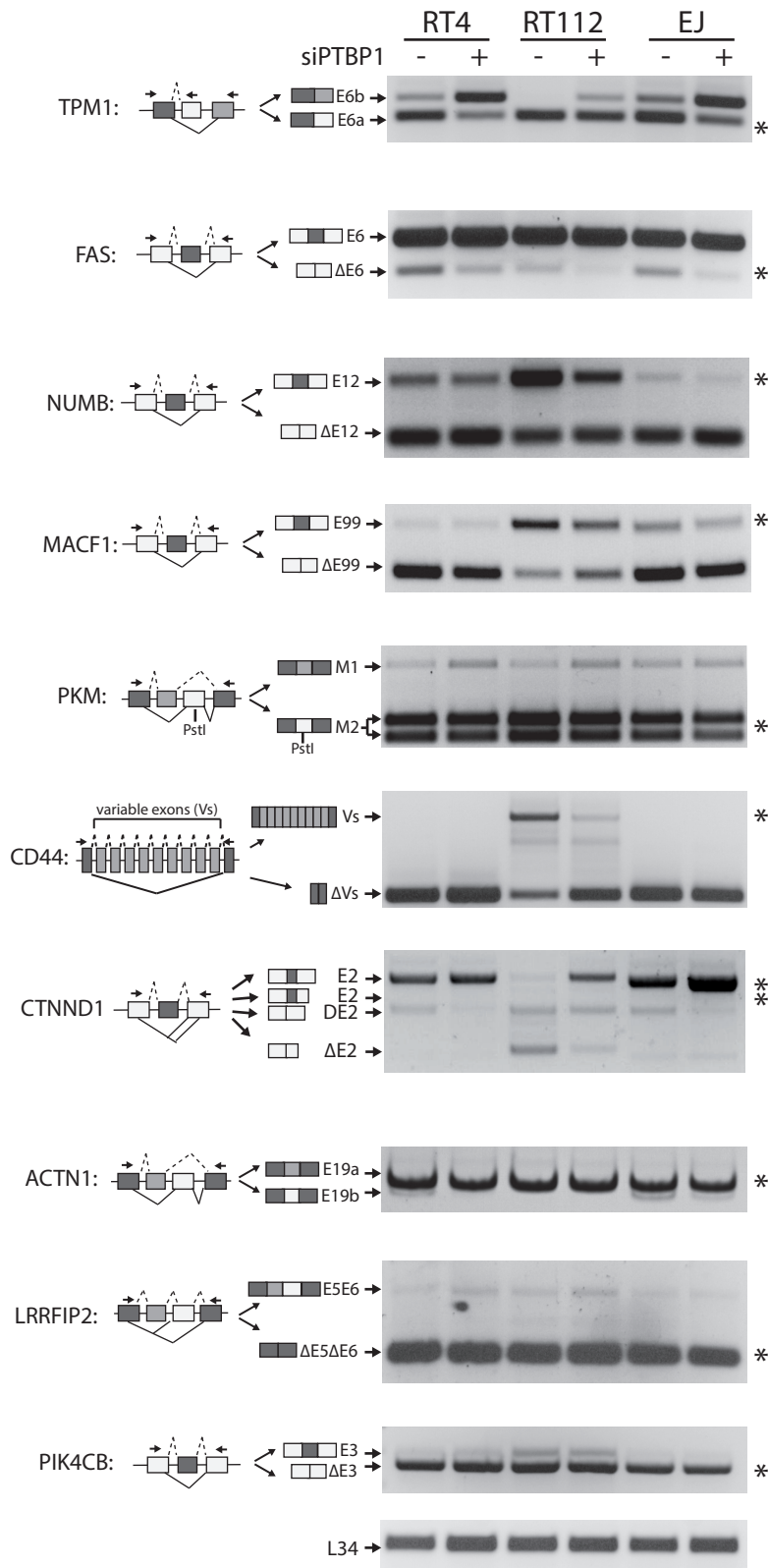


Figure 3

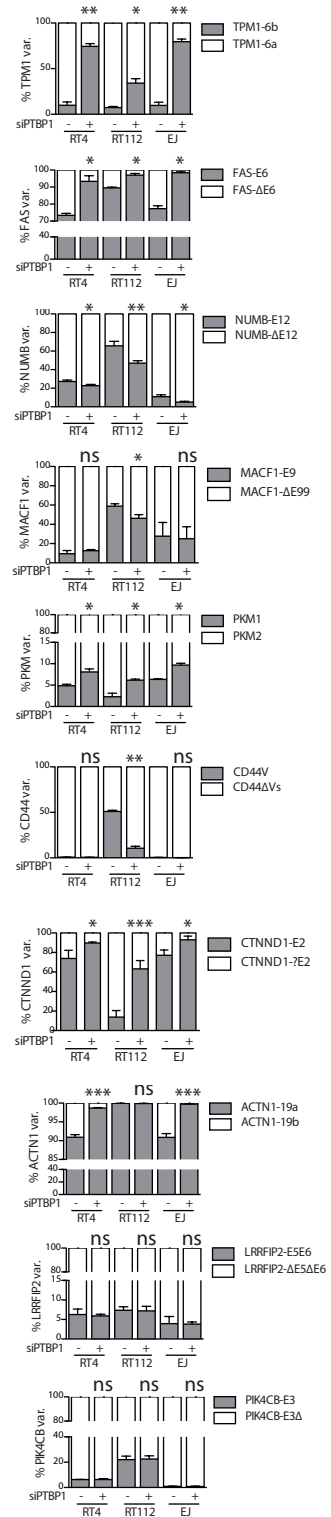
A

PTBP1 target exons

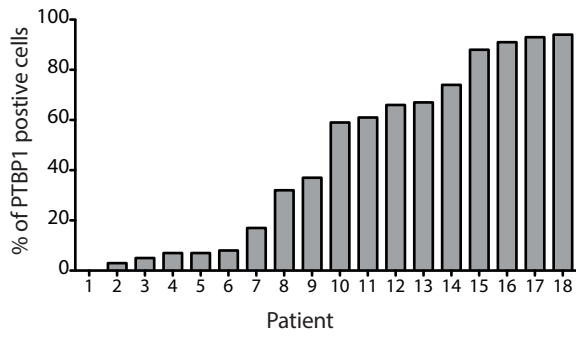
Control exons



B



A



B

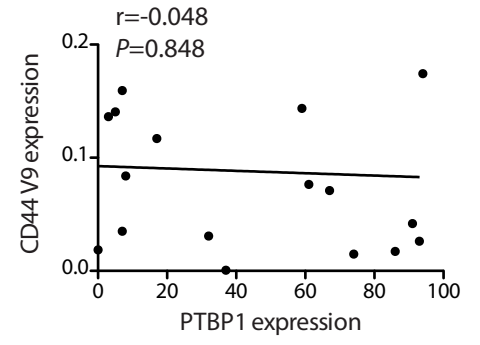
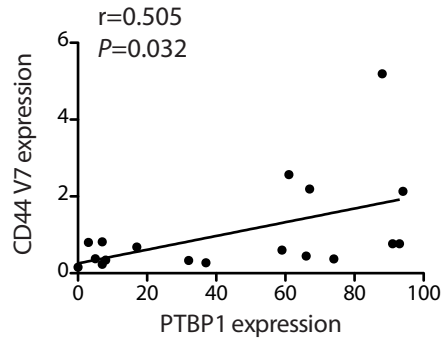
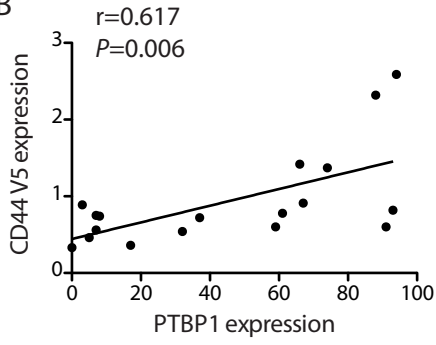


Figure 5

Clinical Cancer Research

The splicing factor PTBP1 promotes expression of oncogenic splice variants and predicts poor prognosis in patients with non-muscle invasive bladder cancer.

Pamela Bielli, Valentina Panzeri, Rossano Lattanzio, et al.

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