T-box transcription factor Brachyury is associated with prostate cancer progression and aggressiveness

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Statement of Translational Relevance

There is an emerging interest and demand to discover new robust biomarkers of prostate cancer (PCa) development and prognostic. The presence of embryonic T-box transcription factor Brachyury has been recently associated with cancer aggressiveness and metastasis. Currently, the role of Brachyury in PCa tumorigenesis is unknown. Using a large cohort of human prostate tissues with different malignancy grades (normal, intraepithelial lesions, primary tumors and metastasis), in silico data, and in vitro studies, we provide the first evidence of aberrant Brachyury activation in primary and metastatic PCa and its clinical relevance. Additionally, we found that Brachyury nuclear expression predicts invasive and metastatic PCa behavior. Herein, we suggest Brachyury as a novel biomarker of PCa metastasis and a potential therapeutic target for advanced PCa patients.
Abstract

Purpose: Successful therapy of prostate cancer (PCa) patients is highly dependent on reliable diagnostic and prognostic biomarkers. Brachyury is considered a negative prognostic factor in colon and lung cancer; however, there are no reports on Brachyury’s expression in PCa.

Experimental design: In this study we aimed to assess the impact of Brachyury expression in prostate tumorigenesis using a large series of human prostate samples comprising benign tissue, PIN lesions, localized tumor and metastatic tissues. The results obtained were compared with what can be inferred from the Oncomine database. Additionally, multiple in vitro models of PCa were used to dissect the biological role of Brachyury in PCa progression.

Results: We found that Brachyury is significantly overexpressed in PCa and metastatic tumors when compared to normal tissues, both at protein and mRNA levels. Brachyury expression in the cytoplasm correlate with highly aggressive tumors, while the presence of Brachyury in the nucleus is correlated with tumor invasion. We found that Brachyury-positive cells present higher viability, proliferation, migration and invasion rates than Brachyury-negative cells. Microarray analysis further showed that genes co-expressed with Brachyury are clustered in oncogenic-related pathways, namely cell motility, cell cycle regulation and cell metabolism.

Conclusions: Collectively, the present study suggests that Brachyury plays an important role in PCa aggressiveness and points, for the first time, to Brachyury as a significant predictor of poor PCa prognosis. Our work paves the way for future studies assessing Brachyury as a possible PCa therapeutic target.
Introduction

Prostate cancer (PCa) is the most common malignancy in men and the second leading cause of cancer-related deaths worldwide. In the USA, PCa is the leading cause of cancer-related mortality (1). Despite advances in prevention and early detection, refinements in surgical techniques and improvements in adjuvant radiotherapy and chemotherapy, metastasis is a frequent event that hinders patients’ cure. One important mechanism that governs cancer cell invasion and further metastasis is cellular epithelial-mesenchymal transition (EMT) (2). The EMT is a complex process that involves downregulation of epithelial markers, such as E-cadherin, and upregulation of mesenchymal markers such as Snail, Slug and N-cadherin, among other alterations. These lead to loss of epithelial cell polarity and acquisition of more motile and invasive phenotypes, promoting cancer cell dissemination into distant sites (3).

The T-box protein Brachyury is a transcription factor required for mesoderm specification during embryo development (4) which is widely expressed in notochord cells and plays a pivotal role in notochord development (5). Recently, Brachyury was associated with tumor aggressiveness in several tumor types (6-11) and found to be a significant predictor of poor prognosis in early colon cancer (8) and lung cancer (6). In vitro studies suggested that these associations are driven by EMT, accomplished by increased migratory and invasion capacity (12-14) and increased cancer stem cell features (10,11). Different studies have reported divergent effects of Brachyury expression on cell proliferation. In lung cancer cell lines it was demonstrated that Brachyury blocks cell cycle progression and, therefore, mediates tumor resistance (15). However, in adenoid cystic carcinoma cells Brachyury promoted tumor growth and
metastasis formation \textit{in vivo} (11). Therefore, despite the described oncogenic role of Brachyury, some authors suggest that it can also act as a tumor suppressor gene (16).

A recent \textit{in vitro} study showed that Brachyury overexpression promoted cell invasion in PCa, probably mediated by TGF-\(\beta\)1 production (13). However, knowledge on the role of Brachyury in PCa progression remains very limited. In the present work, we investigated the clinical impact of Brachyury expression in a well-characterized cohort of human PCa samples and evaluated its biological role in PCa cell proliferation and invasiveness. We report that Brachyury is overexpressed in primary PCa and metastatic tissues and that Brachyury expression is correlated with classic parameters of PCa progression and aggressiveness. We also provide data that suggests Brachyury as a therapeutic target in PCa treatment.
Materials and Methods

Tissue samples

Prostate tissues were obtained from 480 patients with a 64 years old median age (range 46-74), who performed radical prostatectomy as primary therapy (no preceding hormonal or radical therapy) from 1993 to 2010 at Centro Hospitalar do Porto and Centro Hospitalar do Alto Ave-Guimarães, Portugal. The series included a total of 211 non-neoplastic tissue, 143 high-grade prostate intraepithelial neoplasia (PIN) lesions and 409 primary prostate carcinomas (PCa). High-grade PIN lesions and non-neoplastic tissues were obtained from tumor adjacency. Thirteen normal samples were obtained from patients undergoing radical cystoprostatectomy for transitional cell carcinoma of the bladder. Nine metastatic tissues were obtained from patients who performed biopsies for metastatic prostate cancer. Formalin-fixed and paraffin embedded tumors and clinicopathological data were retrieved from the files of the Department of Pathology of both Hospitals. Tumors were staged using the 2010pTNM AJCC classification (17) and graded using the Gleason grading system 2005 (18). Samples were organized into tissue microarray (TMAs) as previously described (19). The histological features of the sampled areas were representative of the final Gleason score for the case. The study was previously approved by Local Ethical Review Committee of Centro Hospitalar do Porto (Ref. no. 017/08 - 010-DEFI/015-CES).

Cell Lines and Cell Culture

Five human prostate cell lines representing in vitro models of PCa progression and aggressiveness, PNT2, 22RV1, LNCaP, PC3 and DU145 (ATCC-American Type Culture Collection, MD, USA), were grown in RPMI-1640 medium supplemented with 10% Fetal
Bovine Serum (FBS) (GIBCO®, Invitrogen) and 1% Penicillin-Streptomycin (P/S) (GIBCO®, Invitrogen). PNT2 is a normal prostate cell line, 22RV1 is a prostate epithelial carcinoma cell line, LNCaP is derived from lymph node metastasis and is hormone-sensitive, and DU145 and PC3 cell lines are derived from brain and bone metastasis, respectively, and represent poorly differentiated tumors.

**Brachyury overexpression and knockdown in prostate cancer cell lines**

22RV1 and DU145 cell lines were transfected with full-length human Brachyury in pcDNA4/TO vector, thus designated pcBrachyury. The empty vector (designated 4/TO) was used as control (12). Stable 22RV1 and DU145 cell pools with pcBrachyury expression were obtained following treatment with Zeocin 50 μg/ml (Invitrogen). PC3 cells were transfected with Brachyury-specific shRNA construct (shBrachy.1) or empty vector alone (pLKO.1) (Sigma-Aldrich) using X-tremeGENE HP transfection reagent (Roche) as recommended by the manufacturer. Stable PC3 cells with depleted endogenous Brachyury expression were obtained following treatment with 5 μg/ml of puromycin (Sigma Aldrich).

**Expression analysis by semiquantitative RT-PCR**

Total RNA was extracted from cell lines using TRIzol® Reagent (Invitrogen S.A.). One μg of RNA was reverse-transcribed using Phusion RT-PCR Kit (Finnzymes), as recommended by the manufacturer. The primers used are presented in Supplementary Table S1. No amplification was obtained when RNA was mock-transcribed without adding reverse transcriptase.

**Western blot analysis**
Cells were lysed in buffer containing 50 mM Tris pH 7.6-8, 150 mM NaCl, 5 mM EDTA, 1 mM Na3VO4, 10 mM NaF, 10 mM NaPyrophosphate, 1% NP-40 and 1/7 of protease cocktail inhibitors (Roche). Proteins were resolved on standard 12% SDS-PAGE gels, transferred onto nitrocellulose membranes and probed with antibody against Brachyury (AF2085, R&D Systems) and GAPDH (sc-69778, Santa Cruz Biotechnology, Inc) at 4°C overnight. Blot detection was done by chemiluminescence (ECL Western Blotting Detection Reagents, GE Healthcare) using Chemidoc (Bio-Rad).

**Immunofluorescence microscopy**

Cells were plated on glass coverslips placed into 12-well plates and allowed to adhere overnight. Cells were fixed with 4% paraformaldehyde (PFA) in PBS1X, washed and permeabilized with 0.1% Triton X-100. Then, cells were blocked in 10% FBS, labeled for 1 hour at room temperature with primary anti-Brachyury antibody (sc-20109, Santa Cruz Biotechnology, Inc), washed and incubated at room temperature for 1 hour with a secondary anti-rabbit Alexa-488 antibody (Invitrogen-Molecular Probes). Coverslips were mounted on microscope slides with Vectashield® Mounting Medium with DAPI (Vector Laboratories). Digital images were recorded with Olympus BX61 (Olympus Corporation).

**Cell viability and proliferation assays**

Colony formation assays were used to assess the survival capacity of 22RV1, DU145 and PC3 cells with and without Brachyury. 1x10³ cells/well were seeded into 6-well plates. After 15 days of culture, colonies formed were fixed in 4% PFA, washed, stained with 0.05% crystal violet and manually counted.
MTS and BrdU assays were used to evaluate the viability and proliferation capacity over time. 2x10^3 cells/well for 22RV1 and 1x10^3 cells/well for DU145 and PC3 were plated into 96-well plates in triplicate and allowed to adhere overnight. After 6 hours of starvation (RPMI only), viable or proliferative cells were quantified using the Cell Titer96 Aqueous cell proliferation (MTS, Promega) or Cell Proliferation ELISA, BrdU (colorimetric, Roche Applied Science) assay and this was the value for time 0. After 24, 48 and 72 hours, cell viability and proliferation were again assessed. The results were calibrated to the starting value (time 0h, considered as 100% of viability/proliferation) as previously described (20).

**Wound healing migration assay**

Cells were seeded in 12-well plates and cultured to at least 95% of confluence. Monolayer cells were washed with PBS1X and scraped with a plastic pipette tip and then incubated with fresh RPMI medium. The “wounded” areas were photographed by phase contrast microscopy at different time points. The relative migration distance was calculated as described (20,21).

**Matrigel invasion assay**

Matrigel invasion assays were performed using 8 µm-pore size BD BioCoatTM Matrigel Invasion Chambers (BD Biosciences). Briefly, after rehydration with RPMI, 10%FBS the upper compartment of the chamber received 2.5x10^5 cells/well grown in RPMI only, whereas the lower compartment contained fresh medium supplemented with 10% FBS and 10 ng/mL of EGF (Prepotech). After 22 hours of incubation, the upper surface of the filter was washed with PBS 1X and fixed with 4% PFA. Then, residual cells were cleared with a cotton swab, the filter washed with PBS 1X and invasive cells attached
to the lower filter surface were mounted in Vectashield® Mounting Medium with DAPI (Vector Laboratories). Images were recorded on an Olympus BX61 microscope (Olympus Corporation) and invasive cells counted using ImageJ software.

**Immunohistochemistry analysis**

Histological slides with 4 μm-thick tissue sections were subjected to immunohistochemistry analysis according to the streptavidin-biotin peroxidase complex system (UltraVision Large Volume Detection System Anti-Polyvalent, HRP; LabVision Corporation), using the primary antibody raised against Brachyury (diluted 1:200; sc-20109, Santa Cruz Biotechnology, Inc) or against AMACR (diluted 1:50; 504R-16, Cell Marque). CD44 staining was performed using an anti-human CD44 antibody (diluted 1:100; 156-3C11, AbD Serotec, Dusseldorf), and detected using Vectastain Universal Elite ABC kit PK-6200 (Vector Laboratories). The negative control was treated identically but with omitted primary antibody. Sections were scored in a double-blind fashion for cytoplasm expression following a semi-quantitative criterion based on the intensity (0=negative, 1=weak, 2=moderate, 3=strong) and percentage of cells stained (0, 0% of immunoreactive cells; 1, <25% of immunoreactive cells; 2, 25–50% of immunoreactive cells; and 3, >50% of immunoreactive cells). Both components were considered for an overall semiquantitative staining score (range from 0 to 6). Samples with scores 0, 1 and 2 were considered negative and those with scores 3, 4, 5 and 6 were considered positive. Tissues sections were separately evaluated for expression in the nucleus (≥25% nuclear staining was considered positive and cases with <25% of nuclear staining were considered negative).

**In silico analysis of Brachyury expression in Oncomine database**
Brachyury mRNA expression was assessed in seven PCa datasets [Latulippe (22), Varambally (23), Grasso (24), Taylor (25), Glinsky (26), Yu (27), TCGA (28) and Arredouani (29)] from Oncomine database (30,31). Categorization of Brachyury-positive and Brachyury-negative PCa patients was based on the Log2 median-centered intensity values of Brachyury probes per study and a linear model was fitted to estimate the association significance. Patient samples in each study with Brachyury expression values greater than its median intensity were grouped as Brachyury positive and others were grouped as Brachyury negative. Brachyury expression was further correlated with corresponding patient clinical data available.

Microarray co-expression studies were extracted from the Oncomine database. Microarray expression profiles were clustered by functional importance and signaling pathways using DAVID v6.7 bioinformatic tool (The Database for Annotation, Visualization and Integrated Discovery) (32,33).

**Statistical analysis**

Univariate (chi-square test; \( \chi^2 \)-test) and multivariate analysis (linear regression model) were used to assess the correlations between Brachyury expression and clinicopathological features from primary specimens. Pearson’s test was used to evaluate the correlation between gene expression profiles. Simple comparisons between two different conditions were analyzed using Student’s t test and, for comparison of two conditions over time, we used the 2way ANOVA analysis (Bonferroni post-test). The statistical analysis was performed using SPSS software (version 19.0) or using Prism GraphPad software (version 5.0a). The level of significance in the statistical analyses is indicated as \(*=p<0.05, **=p<0.01\) or
***=p<0.001.
Results

**Brachyury protein is overexpressed in prostate cancer and prostatic intraepithelial neoplasia lesions**

Brachyury protein expression was assessed by immunohistochemistry in a series of 784 prostate tissues, including normal tissues, PIN lesions, primary PCa samples with different Gleason scores and PCa metastasis. Figure 1 shows representative results of intensity scores observed for Brachyury expression. Normal prostate gland and adjacent non-neoplastic tissues presented absence or low levels of Brachyury staining when compared with neoplastic tissues (Fig. 1A, Supplementary Fig. S1) and were therefore clustered in a single group, designated non-neoplastic tissues. Brachyury was expressed in the nuclei and/or cytoplasm of epithelial cells in non-neoplastic tissues, PIN lesions, PCa and metastatic tissues (Fig. 1B-D, Supplementary Fig. S1). Overall, the number of cases presenting cytoplasm protein expression increased from non-neoplastic, to PCa and PIN lesions and to metastasis (33.9%, 55.2%, 61.5%, and 100% of positive cases, respectively; \( p < 0.001; \text{Fig. 2A} \)). Brachyury nuclear staining was present in a comparable number of cases in non-neoplastic (25.0%), PIN lesions (38.6%) and PCa cases (25.4%), in contrast to 100% of metastatic tissue samples (\( p < 0.001; \text{Fig. 1vi and Fig. 2A} \)). Interestingly, Brachyury was also detected in the stroma (Supplementary Fig. S1) with a significant reduction of stroma-positive cases from non-neoplastic tissues (52.6%), to PIN lesions (44.2%) and to PCa (14.2%) (\( p < 0.001; \text{Fig. 2A} \)), indicating a possible role for Brachyury in PCa tumor tissue microenvironment.

Heat map analysis of overall Brachyury protein expression showed that Brachyury is remarkably overexpressed in PIN, PCa and metastatic prostate tissues when compared
with non-neoplastic tissues (Fig. 2B). The higher expression profile was found in metastasis with scores consistently ≥4.

**Brachyury protein overexpression is associated with poor prognosis in prostate cancer**

The clinical impact of Brachyury protein expression levels was further explored in our cohort of 409 primary PCa. Univariate outcome analysis showed that Brachyury positive cases (scores ≥3) are significantly ($p<0.001$) associated with the PCa biomarker alpha-methylacyl-CoA racemase (AMACR; Supplementary Table S2). Brachyury positive cases correlated with highly undifferentiated PCa tumors ($p=0.007$, Table 1) and, concordantly, a strong tendency to be associated with stem cell marker CD44 was observed ($p=0.054$; Supplementary Table S2). Importantly, Brachyury protein levels increased with the Gleason score ($p<0.027$, Table 1; $p<0.01$, Supplementary Fig. S2). In order to evaluate the clinical impact of the presence of Brachyury in the nucleus, a comparison between Brachyury nuclei-positive and nuclei-negative in PCa positive cases was performed (non-neoplastic tissues, n=76; PIN lesion, n=88; PCa, n=228; metastasis, n=9). Primary PCa tumors with nuclear Brachyury staining were significantly associated with perineural invasion ($p=0.046$) and with capsular invasion ($p=0.025$) (Table 1), which is in agreement with predominant nuclear expression in metastasis (Fig. 1 and Fig. 2). Since Gleason scores and pT stage are known prognostic biomarkers, we performed multivariate analysis to determine whether high Brachyury expression has an independent statistical value. We observed that Brachyury is
significantly associated with capsular invasion (p=0.027, Table 1) on primary PCa samples.

No significant correlations were found for Brachyury staining in stroma with clinicopathological parameters by univariate analysis (Table 1). Yet, the multivariate analysis showed a significant association with capsular invasion (p=0.030; Table 1), indicating the possible role of Brachyury on tumor microenvironment.

Altogether, these data suggest that high Brachyury levels are associated with patient’s poor outcome and indicate that nuclear Brachyury staining in PCa is an independent prognostic factor.

*In silico* validation of the role of Brachyury expression in prostate cancer aggressive behavior

To corroborate our findings, we extended the analysis to microarray profiling datasets of PCa tissues available on the Oncomine database (30, 31). Brachyury mRNA expression was analyzed in six independent PCa datasets [Latullipe (22), Varambally (23), Grasso (24), Taylor (25), Yu (27) and Arredouani (29)] comprising a total of 97 normal prostate gland, 304 PCa and 83 PCa metastasis samples. We found that Brachyury was significantly overexpressed in prostate tissues in multiple microarray cancer profiling datasets, in particular in metastatic prostate cancer (Fig. 2C). This was concordant with our protein analysis reported above. Importantly, although multiple probes were used to determine Brachyury mRNA levels in these datasets (23996_at,
206524_at, A_24_P63642, 7679), they all consistently showed that Brachyury overexpression is a common event in primary and metastatic PCa (Fig. 2C).

We next assessed the impact of Brachyury in PCa prognosis at the mRNA level, exploring the microarray profiling datasets of localized prostate tumors with clinical data from Oncomine (Table 2). Univariate statistical analysis revealed that high levels of Brachyury expression correlated with higher (>7) Gleason scores for Taylor (25), Glinsky (26) and Latulippe (22) datasets (p=0.043; p=0.042 and p=0.049, respectively; Table 2). In agreement with protein immunohistochemistry levels, the percentage of Brachyury positive cases directly increased with the Gleason score (p<0.05; Supplementary Fig. S2). Additionally, high Brachyury mRNA levels correlated with pT [Yu(27), p=0.033] and N stage [Taylor (25), p=0.043], biochemical recurrence [Taylor (25), p=0.048], capsular invasion and extraprostatic extension [Glinsky (26), p=0.002 and p=0.007, respectively] (Table 2). A similar tendency could also be observed in the other datasets. In the multivariate analysis, we found that high Brachyury mRNA levels still correlate with capsular invasion and extraprostatic extension [Glinsky (26), p=0.001 and p=0.032, respectively] and with biochemical recurrence [TCGA (28), p=0.004] (Table 2).

**Prostate cell lines recapitulate Brachyury expression profiles of human PCa tissues**

To explore the biological role of Brachyury in PCa aggressiveness, five prostate cancer cell lines (PNT2, 22RV1, LnCaP, PC3 and DU145), representative of different degrees of PCa progression, were screened for Brachyury expression by semi-quantitative RT-PCR and western blot analyses. Brachyury protein sub-cellular localization was additionally
evaluated by immunofluorescence. We observed an absence of Brachyury at both mRNA and protein levels in the non-malignant prostate cell line (PNT2) and in the primary PCa cell line (22RV1) (Supplementary Fig. S3A). In contrast, the metastatic cell lines LNCaP and PC3 showed strong nuclear and cytoplasm Brachyury expression both at mRNA and protein levels (Supplementary Fig. S3A). The metastatic DU145 cell line was negative for mRNA by conventional RT-PCR, but still exhibited low levels of nuclear protein expression (Supplementary Fig. S3A). These findings indicate that prostate cell lines are good models to study the functional role of Brachyury in prostate cancer cells, since they recapitulate the expression profiles found in human clinical samples.

**Brachyury promotes prostate cancer aggressiveness in vitro**

To address whether the modulation of Brachyury expression influences the tumorigenic properties of PCa cells, Brachyury was overexpressed in primary (22RV1) and metastatic (DU145) PCa cell lines. Successful ectopic overexpression was obtained upon transfection of both cell lines with the pcBrachyury expression vector and Brachyury protein exhibited nuclear localization (Supplementary Fig. S3B). To investigate the effect of Brachyury inhibition, a specific short-hairpin clone (shBrachy.1) was used to deplete Brachyury in a positive metastatic prostate cell line (PC3) (Supplementary Fig. S3B).

We initially studied the biological role of Brachyury on PCa cell viability and proliferation (Fig. 3A; Supplementary Fig. S4A). pcBrachyury prostate cells had a significant ($p<0.05$) viability advantage over time (MTS assay) compared with the cells transfected with the empty vector (4/T0). Colony formation assays revealed a
significant (p<0.05) increase in the number of the colonies formed in the pcBrachyury transfected cells when compared to the control cells (Fig. 3A, Supplementary Fig. S4A).

The opposite findings were obtained with Brachyury depletion in shBrachy.1-PC3 cells (p<0.05; Fig. 3A). To determine whether this viability advantage was due to higher proliferation rates, we analyzed BrdU incorporation during S phase of the cell cycle. The presence of Brachyury, whether endogenous or exogenously overexpressed, promoted higher rates of proliferation over time (p<0.05; Fig. 3A, Supplementary Fig. S4A). We further performed wound migration and matrigel invasion assays in the transfected cell lines and observed that both 22RV1 and DU145 pcBrachyury cells had a higher migratory capacity over time and increased cell invasion capability comparing with the empty vector cells (p<0.05; Fig. 3B, Supplementary Fig. S4B). When Brachyury was depleted in an endogenously positive cell line, we were able to attenuate the aggressive behavior (p<0.05; Fig. 3B).

To characterize the molecular players underlying prostate aggressiveness in vitro, we studied the expression profile of some key genes involved in EMT, migration and stemness processes. We observed that Brachyury expression was associated with a decrease of the epithelial marker E-cadherin and concomitant increased expression of mesenchymal genes (N-cadherin, fibronectin, and Snail), as well as upregulation of metalloprotease MMP14 (Supplementary Fig. S5). Concordantly with immunohistochemistry analysis in human PCa (Table S2), Brachyury overexpression was associated with an increased expression of the stem cell marker CD44 (Supplementary Fig. S5).
Genes co-expressed with Brachyury in microarray analyses are associated with tumorigenic clusters

We clustered the genes co-expressed with Brachyury in prostate tissues available at Oncomine database (30,31) by their functional role and importance in signal transduction pathways using the DAVID bioinformatic tool (Fig. 4A). We found that the majority of genes co-expressed with Brachyury were functionally clustered in the categories of immune response, cell membrane/receptor activity, development, cell motility and chemotaxis, pathways in cancer and response to hormone stimulus. A sub-analysis by KEGG signaling pathways revealed that Brachyury co-expressed genes are grouped in pathways associated with higher aggressiveness, namely, pathways in cancer, positive cell cycle regulation and immune response (Fig. 4B). These analyses strongly point to a role of Brachyury, not only in cell migration and invasion, but also as a regulator of the cell cycle and in cancer microenvironment metabolism. Importantly, we found that the levels of Brachyury expression in PCa tissues are directly correlated with those of IL-8 and TGF-β1 (Fig. 4C), which are involved in EMT and cancer microenvironment modulation, as previously described (13,14). Accordingly, there is a correlation of Brachyury expression with the expression of genes associated with EMT process (like fibronectin) and migration (MMP14; MMP24) (Fig. 4C) that support our expression analysis (Supplementary Fig. S5).
Discussion

The T-box transcription factor Brachyury was initially discovered for its role in mouse mesoderm development and differentiation (34). This involves massive conversion of epithelial cells into migratory and invasive mesenchymal cells during gastrulation via a process known as EMT (2,4). Several reports have demonstrated that EMT is critical for PCa progression, as acquisition of mesenchymal features may favor dissemination and resistance to therapy (35). High levels of Brachyury have previously been reported in various types of cancer (5-9,11) and a phase I clinical trial of a vaccine targeting Brachyury-positive tumors (GI-6301) is currently underway (36,37). Although PCa is a leading cause of cancer-related deaths in men worldwide, a characterization of Brachyury biological role in prostate tumorigenesis is missing.

Our study reports for the first time that the transcription factor Brachyury is aberrantly overexpressed across prostate malignancy and, in particular, nuclear Brachyury staining are associated with prostate invasion and prostate metastatic tissues. Our findings indicate Brachyury as an independent prognostic factor in PCa. The role of Brachyury nuclear staining in metastasis was demonstrated in other tumor types, such as colorectal, lung cancer and oral squamous cell carcinoma (6-9). Herein, we also found that cytoplasm immunostaining in PCa is associated with PCa biomarker AMACR and with highly aggressive tumors. The role of Brachyury in the cell cytoplasm remains to be elucidated, yet, we can hypothesize that it interacts with other proteins and in this way regulates cell behavior in a non-transcription manner.

It has been shown that Brachyury expression can influence tumor microenvironment through the release of soluble factors that could induce adjacent epithelial tumor cells
to undergo an EMT and acquire metastatic potential (14). In PCa, the activation and secretion to the extracellular environment of soluble factors that mediate the cross-talk between tumor cells and tumor stroma, such as interleukins and growth factors, has been reported to play a role in tumor progression (13,14,38-40). Our analysis confirm a previously result that demonstrated that Brachyury increases the expression and secretion of TGF-β1 in a prostate cell line (13). However, the influence of Brachyury expression in stromal cells has not yet been characterized. To our knowledge, we provide the first evidence for decreased Brachyury expression in stromal cells with prostate malignancy, at variance with the reported upregulation in tumor cells. Therefore, we can hypothesize that Brachyury has different roles in stromal and tumor cells and that it could be involved in the regulation of tumor microenvironment. Additionally, we found that the majority of Brachyury co-expressed genes are involved in immune or metabolic processes.

By Brachyury overexpression and downregulation in prostate cancer cell lines, we demonstrated its role in tumor cell migration and invasion, as well as in cell viability and proliferation. Our findings were further corroborated by an in silico analysis with multiple genes functionally clustered in pathways related with cell motility and cell proliferation. A study performed by Shomoda et al. demonstrated that ablation of Brachyury in ACC cells decreased the number of metastasis and tumor size in vivo (11). Moreover, depletion of Brachyury in chordoma cells promotes a complete block of cell proliferation (41). An opposite role for Brachyury in cell proliferation was demonstrated in lung and colorectal cell lines by Huang and collaborators, where Brachyury blocks cell cycle progression and mediates tumor resistance to conventional
antitumor therapies (15). Therefore, it can be deduced that the role of Brachyury may be tissue-specific or cell type-dependent.

Brachyury seems to be a key driver of EMT in various human tumors by increasing expression of genes such as Slug, Snail, MMPs, IL-8 and TGF-β1 (6,9,13-15, and current study). A possible link between cells undergoing EMT and cells with “stem cell-like” properties was recently described (42). The role of Brachyury in conferring stemness properties was already demonstrated in colorectal cancer cells (10) and in ACC cells (11). The present study shows that Brachyury is more represented in CD44⁺ prostate tissues and ectopic Brachyury overexpression in vitro promotes CD44 expression. Since CD44⁺ prostate tumors are more resistance to the currently employed therapies (43,44), we speculate that Brachyury could have a role in PCa therapy resistance. Future studies are warranted to elucidate this hypothesis.

In conclusion, the present work reports increased levels of Brachyury expression in localized and metastatic prostate cancer, with clinicopathological significance and evidences a role for Brachyury in promoting PCa cell growth and invasion. Our work further suggest new roles for Brachyury in PCa, namely in tumor microenvironment regulation and possibly in immune response. Clinical applicable prognostic biomarkers are needed for clinical management of PCa patients and our study positions Brachyury as a putative independent prognostic biomarker in PCa and a possible therapeutic target for advanced prostate tumor patients.
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Table 1. Correlation between Brachyury sub-cellular localization and clinicopathological features in PCa tissues by univariate and multivariate analysis.

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<tr>
<th>Clinical parameters</th>
<th>Overall staining</th>
<th>Nuclear staining in positive cases</th>
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* For multivariate analysis Gleason score and pT stage were used as variables to determine whether high Brachyury indicates poor outcome.
Table 2. Correlation of Brachyury microarray expression profiles with clinicopathological features available in different datasets from Oncomine database by univariate and multivariate analysis.

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<th>Data set</th>
<th>Taylor (25)</th>
<th>Glinsky (26)</th>
<th>TCGA (28)</th>
<th>LaTulippe (22)</th>
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<td>p value</td>
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* For multivariate analysis Gleason score, pT and N stages were used as variables to determine whether high Brachyury indicates poor outcome. NA, not-available.
Figure Legends

**Figure 1. Brachyury expression in non-neoplastic tissues, PIN lesions, PCa and metastatic tissues.**

Brachyury is absent or expressed at low levels in non-neoplastic tissues (A, normal gland). Primary PCa Brachyury-positive cases can exhibited only cytoplasm staining (B), or both cytoplasm and nuclear staining (C); Metastatic lesion showing both cytoplasm and nuclear (D). Magnification of 200X (A) and 400X (B,C and D).

**Figure 2. Brachyury is overexpressed in PCa and metastatic tissues.**

A) Representation of Brachyury-positive cases according to Brachyury localization; left panel, overall score for cytoplasm staining; central panel, presence in nucleus; right panel, presence in stromal cells. B) Heatmap of protein levels in tissue microarray prostate samples (range 0-6). There is a predominant blue staining (negative, score<3) in normal tissues and orange/red staining (positive, score≥3) in PIN, PCa and metastasis tissues. Each column represents a single case and it is possible verify the respective normal adjacent tissue or PIN lesion of a specific PCa case. In the majority of the cases an increased expression from normal to PIN and to PCa can be appreciated in the same patient. C) Analysis of microarray expression data for Brachyury levels from the Oncomine database. Log2 median-centered ratio expression is present for six different datasets [Yu (27), Varambally (23), Grasso (24), Taylor (25), Latullipe (22) and Arredouani (29)] representing 4 different probes for Brachyury detection (34996_at, ...
206524_at, A_24_P63642 and 7679). **Brachyury** is commonly overexpressed in PCa tissues and PCa metastasis. *, p<0.05; **, p<0.01; ***, p<0.001.

**Figure 3. Evaluation of biological role of Brachyury in prostate cancer cell lines.**

**A)** Effect of Brachyury on viability and proliferation of prostate cells (22RV1 and PC3) evaluated by MTS, colony formation and BrdU assays. **B)** Wound healing and matrigel invasion assay were used to evaluate the role of Brachyury in migration and invasion, respectively. The presence of Brachyury correlated with increased cell viability, proliferation, migration and invasion. Red lines and black bars represent Brachyury-positive cell lines; Blue lines and white bars, Brachyury-negative or -depleted cell lines. *, p<0.05; **, p<0.01; ***, p<0.001.

**Figure 4. In silico analysis of genes co-expressed with Brachyury in PCa.** Microarray expression profiles of Brachyury co-expressed genes were clustered by functional role and signaling pathways using DAVID *in silico* tool. **A)** Left panel represents the functional clusters organized by enrichment score and **B)** right panel represents the KEGG signaling pathway analysis. Brachyury co-expressed genes are associated with pathways involved in tumor aggressiveness, namely in immune cell response, positive cell cycle regulation, cell motility and chemotaxis. **C)** *In silico* analysis indicates an inverse correlation between Brachyury and epithelial marker E-cadherin and a direct correlation with several genes involved with EMT (*fibronectin, MMP14, MMP24, Snail, IL-8* and *TGF-β1*). NS, not-significant.
Figure 2

A

B

C
Figure 3

(A) MTT assay

(B) AlamarBlue™ assay
Clinical Cancer Research

T-box transcription factor Brachyury is associated with prostate cancer progression and aggressiveness

Filipe Pinto, Nelma Pertega-Gomes, Marcia S Pereira, et al.

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