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Brachyury oncogene is a prognostic factor in high-risk testicular germ cell tumors

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SUMMARY

The T-box transcription factor Brachyury has been considered a cancer-specific marker and a novel oncotarget in solid tumors. Brachyury overexpression has been described in various cancers, being associated with epithelial–mesenchymal transition, metastasis, and poor prognosis. However, its clinical association with testicular germ cell tumor is unknown. We analyzed the expression of Brachyury by immunohistochemistry in a series of well-characterized testicular germ cell tumor samples and at transcript level by *in silico* analysis. Additionally, we aimed to investigate the clinical significance of Brachyury in testicular germ cell tumor. Brachyury cytoplasm immunostaining was present in 89.6% (86/96) of cases with nuclear staining observed in 24% (23/96) of testicular germ cell tumor. Bioinformatics microarray expression analysis of two independent cohorts of testicular germ cell tumors showed similar results with increased levels of *Brachyury* in testicular germ cell tumors and metastasis compared with normal testis. Clinically, Brachyury nuclear staining was statistically associated with lower event-free survival ($p = 0.04$) and overall survival ($p = 0.01$) in intermediate/high-risk testicular germ cell tumors. Univariate analysis showed that Brachyury nuclear subcellular localization was a predictor of poor prognosis ($p = 0.02$), while a tendency was observed by multivariate analysis (HR: 3.56, $p = 0.06$). In conclusion, these results indicate that Brachyury plays an oncogenic role in testicular germ cell tumors and its subcellular localization in the nucleus may constitute a novel biomarker of poor prognosis and a putative oncotarget for intermediate/high-risk testicular germ cell tumor patients.

INTRODUCTION

Brachyury (*T*) is a T-box transcription factor family with a central role in notochord and mesoderm specification (Herrmann *et al.*, 1990). In the last years, Brachyury has been described by us and others to be upregulated in several solid tumors, including chordoma (Vujovic *et al.*, 2006), lung (Roselli *et al.*, 2012), breast (Palena *et al.*, 2014), colorectal (Kilic *et al.*, 2011), prostate (Pinto *et al.*, 2014) cancer, and GIST (Pinto *et al.*, 2016a,b). Importantly, in these tumors, Brachyury has been reported as an independent biomarker of poor prognosis (Kilic *et al.*, 2011; Haro *et al.*, 2013; Palena *et al.*, 2014; Pinto *et al.*, 2014, 2016a,b). Brachyury acts as a key player in the epithelial–mesenchymal transition (EMT) and metastasis formation (Fernando *et al.*, 2010; Imajyo *et al.*, 2012; Shimoda *et al.*, 2012; Du *et al.*, 2014; Palena *et al.*, 2014; Pinto *et al.*, 2014; Shao *et al.*, 2015; Xu *et al.*, 2015), also playing an important role in promoting stem cell properties (Sarkar *et al.*, 2012; Shimoda *et al.*, 2012; Jezkova *et al.*, 2016; Pinto *et al.*, 2016a,b) and resistance to cytotoxic-based therapy (Fernando *et al.*, 2010; Roselli *et al.*, 2012; Pinto *et al.*, 2016a,b). Based on Brachyury oncogenic behavior, an anti-Brachyury vaccine (GI-6301) was developed and is currently in Phase II clinical trial (www.clinicaltrials.gov, 2015 – NCT02383498) for patients with chordoma (Heery *et al.*, 2015).

TGCTs represent the most frequent type of cancer in young men (Siegel *et al.*, 2016). In non-seminomas, the risk is based on serum markers levels, primary site, and extrapulmonary metastasis, according to International Germ Cell Cancer Collaborative Group (IGCCCG) (IGCCCG, 1997). TGCTs present high sensitivity to first-line platinum-based chemotherapy, and the majority of patients with metastatic disease may expect to be highly

responsive, achieving high cure rates, therefore, considered as a model of curable malignancy (Einhorn, 1990; Feldman *et al.*, 2008). However, ~15% of cases develop refractory disease, being the majority of non-seminomas, that fail to be cured following the first-line treatment (Mead *et al.*, 2005; International Prognostic Factors Study *et al.*, 2010). Thus, identification of novel biomarkers and prognostic factors for TGCTs, especially in high-risk group, may be useful for better stratification of patients and potentially may be used as novel therapeutic targets. Brachyury is considered a cancer-specific marker, being absent in normal adult tissues with the exception of testis (Fernando *et al.*, 2010; Hamilton *et al.*, 2015). A recent study also showed that nuclear Brachyury expression is present in germ cell tumors (range between 14% to 74%), yet its clinical–pathological impact was not evaluated (Miettinen *et al.*, 2015).

Therefore, in this study, we performed an immunohistochemistry analysis of Brachyury expression in TGCT and interrogated its clinical impact. Moreover, we extended our findings using *in silico* gene expression analysis of large TGCT datasets. We found that Brachyury is overexpressed in TGCTs, and its subcellular nuclear expression may represent a novel biomarker of poor prognosis in intermediate/high-risk TGCTs and a potential therapeutic target, via GI-6301 anti-Brachyury vaccine.

MATERIALS AND METHODS

Tissue samples

A series of paraffin-embedded TGCT primary tissue samples, composed of 96 TGCTs, was obtained from the Pathology Department of Barretos Cancer Hospital, Barretos, Brazil. The samples were collected prior to the use of any systemic treatment. The study was conducted following the national and institutional ethical policies and was approved by the Barretos Cancer Hospital Ethical Committee (protocol 676/2013). The clinicopathological features of TGCTs across different histological subtypes are presented in Table 1.

Brachyury immunohistochemistry

The immunohistochemistry analyses were performed as previously described (Pinto *et al.*, 2014, 2016a,b). Representative 4-μm-thick 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 (sc-20109: Santa Cruz Biotechnology, Inc). Tumor samples were evaluated for extension (percentage of cytoplasm-positive cells: 0, negative; 1, <25% positive cells; 2, 26–50% positive cells; 3, >50% positive cells) and intensity (0, negative; 1, weak; 2, moderate; 3, strong) of the immunoreactions. The score used for cytoplasm staining was the sum of the extension and the staining intensity. Samples with scores 0–2 were considered negative, and those with scores 3–6 were considered positive. Brachyury nuclear staining was also evaluated and considered negative (<25% of the cells with positive nuclei staining) or positive (≥25% of the cells depicting nuclear staining). The final score of Brachyury staining was the combination of both cytoplasm staining and nuclear staining, negative (negative for both subcellular locations) and positive (positive for cytoplasm and/or nuclear staining).

Table 1 Clinicopathological features of the TGCT patients

Characteristics	*n* (%)
Valid TGCT	96
Age (years)	
Mean (SD)	28.9 [8.0]
Min-max	18–62
Testis side	
Left	46 (48.0)
Right	50 (52.0)
Histology group	
Non-seminoma	71 (74.0)
Seminoma	25 (26.0)
Histology	
Mixed tumor	27 (28.1)
Seminoma	25 (26.0)
Mixed tumor plus teratoma	18 (18.7)
Embryonal carcinoma	11 (11.5)
Yolk sac tumor	07 (7.3)
Immature teratoma	05 (5.2)
Mature teratoma	1 (1.0)
Choriocarcinoma	2 (2.1)
Serum markers (AJCC)	
S0	01 (1.0)
S1	32 (33.3)
S2	25 (26.0)
S3	06 (6.2)
SX	32 (33.3)
Stage (AJCC)	
I	6 (6.2)
IS	12 (12.5)
II	31 (32.3)
III	47 (49.0)
Number of metastasis sites	
0	18 (18.8)
1	44 (45.8)
2	19 (19.8)
≥3	15 (15.6)
First-line chemotherapy	
BEP	77 (80.2)
EP	17 (17.7)
Carboplatin	1 (1.0)
Other	1 (1.0)
Number of first-line chemotherapy cycles	
1	8 (8.3)
2	5 (5.2)
3	29 (30.2)
4	54 (56.2)
Chemosensitivity	
Responsive	68 (70.8)
Refractory	19 (19.8)
Not applicable	9 (9.4)
IGCCCG risk	
Low	46 (47.9)
Intermediate	18 (18.7)
High	13 (13.5)
Not applicable	18 (18.7)
Unknown	1 (1.0)

SD, Standard deviation; AJCC, American Joint Committee on Cancer; IGCCCG, International Germ Cell Cancer Cooperative Group; BEP, Bleomicine, Etoposide and Cisplatin; EP, Etoposide e Cisplatin.

In silico Brachyury expression analysis: Oncomine and TCGA databases

Brachyury expression was assessed in three datasets containing specific information for TGCTs extracted from the Oncomine database (www.oncomine.org, November 2017) (Rhodes *et al.*, 2007) [Korkola (Korkola *et al.*, 2006) and Skotheim (Skotheim *et al.*, 2005)] and from TGCA database (http://cancergenome.nih.gov/) (Cancer Genome Atlas Research *et al.*, 2013). *Brachyury* expression was assessed in a total of 10 normal testis

samples, 272 primary TGCTs, and in six metastases. No relevant clinical information as overall survival, follow-up, treatment, risk, or stage was available for download.

Statistical analysis

Correlations between Brachyury expression and clinicopathological data were performed using the chi-square test or Fisher's exact test. Cumulative survival probabilities were calculated using the Kaplan–Meier method. Differences between survival rates were evaluated by univariate (log-rank test) and multivariate analysis (Cox proportional hazard model). The statistical analysis was performed using SPSS-v20.0. Simple comparisons between two different conditions were analyzed using Student's t-test in Prism GraphPad-v5.0a. The level of significance in the statistical analyses is indicated as $*p < 0.05$, as $**p < 0.01$ or as $***p < 0.001$.

RESULTS

Expression profile of Brachyury in TGCTs

A cohort of 96 TGCTs was submitted to immunohistochemistry analysis of Brachyury. We found positive Brachyury immunostaining, predominantly nuclear, in the seminiferous tubules of normal testis (Fig. 1a). Overall, 89.6% ($n = 86/96$) of TGCTs showed positivity of Brachyury staining (Fig. 1b,c, Table S1). Specifically, 73.3% ($n = 63/86$) of the TGCT-positive tissues depicted Brachyury staining only in the cytoplasm (Fig. 1b), while 26.7% ($n = 23/86$) exhibited prevalently nuclear Brachyury staining, although weakly cytoplasm positivity was also observed (Fig. 1c).

For statistical analysis, TGCT were classified into negative (negative for both cytoplasm and nuclear staining; $n = 10/96$) *vs* positive (positive for both cytoplasm and/or nuclear staining; $n = 86/96$) or based on Brachyury nuclear staining: nuclear-negative (76.0%; $n = 73/96$) vs. nuclear-positive (24.0%; $n = 23/96$) (Tables S1 and S2). A statistical analysis was also carried out based on Brachyury subcellular localization: negative (10.4%; $n = 10/96$), cytoplasmic (65.6%; $n = 63/96$), and nuclear staining (both cytoplasm and nuclear; 24.0%; $n = 23/96$) (Tables S1 and S2). Brachyury positivity and Brachyury nuclear subcellular localization staining were statistically associated with the presence of altered serum markers (AJCC) S1, S2, S3, or Sx (Table S1). No association between Brachyury levels and other clinicopathological variables such as age, histology, stage, number of metastasis, chemotherapy response, or IGCCCG risk was found (Table S1).

We were able to identify peritumoral seminiferous tubules in a subset of cases ($n = 23$, 24%) where germ cell neoplasia *in situ* (GCNIS) was present (Fig. 1d). Nuclear Brachyury staining was positive in 78.3% of those cases. Only six cases (33.3%) were nuclear-positive both in germ cell tumor and GCNIS, and there was no association of nuclear Brachyury staining between GCNIS and germ cell tumors ($p = 1.0$).

In silico microarray analysis showed *Brachyury* mRNA levels are associated with TGCT and metastasis

To extend our study at transcript level, we have performed an *in silico* analysis of *Brachyury* mRNA levels in TGCTs from TCGA and Oncomine databases. Extracted data represents the only three datasets with gene expression data available for TGCTs [TCGA ($n = 156$); Korkola ($n = 110$); Skotheim ($n = 22$)]. RNASeq analysis showed that *Brachyury* is overexpressed in TGCTs (Fig. 2a, TCGA: $n = 156$ samples). In more, two independent datasets with expression data for normal testis samples (Korkola and Skotheim) demonstrate an increased *Brachyury* expression in TGCT and metastasis (Fig. 2b and c). To have statistical

Figure 1 Immunohistochemical images of Brachyury staining in TGCT. (a) Nuclear Brachyury staining in seminiferous tubules of normal testis. (b) Brachyury-positive staining in the cytoplasm in an embryonal carcinoma. (c) Predominantly and increased Brachyury nuclear staining in a mixed tumor. A weak cytoplasm staining was also observed. (d) Nuclear Brachyury staining of malignant spermatogonia in intratubular germ cell tumor. (e) Hematoxylin and eosin staining of intratubular germ cell tumor. Magnification: 400×. TGCT, testicular germ cell tumor.

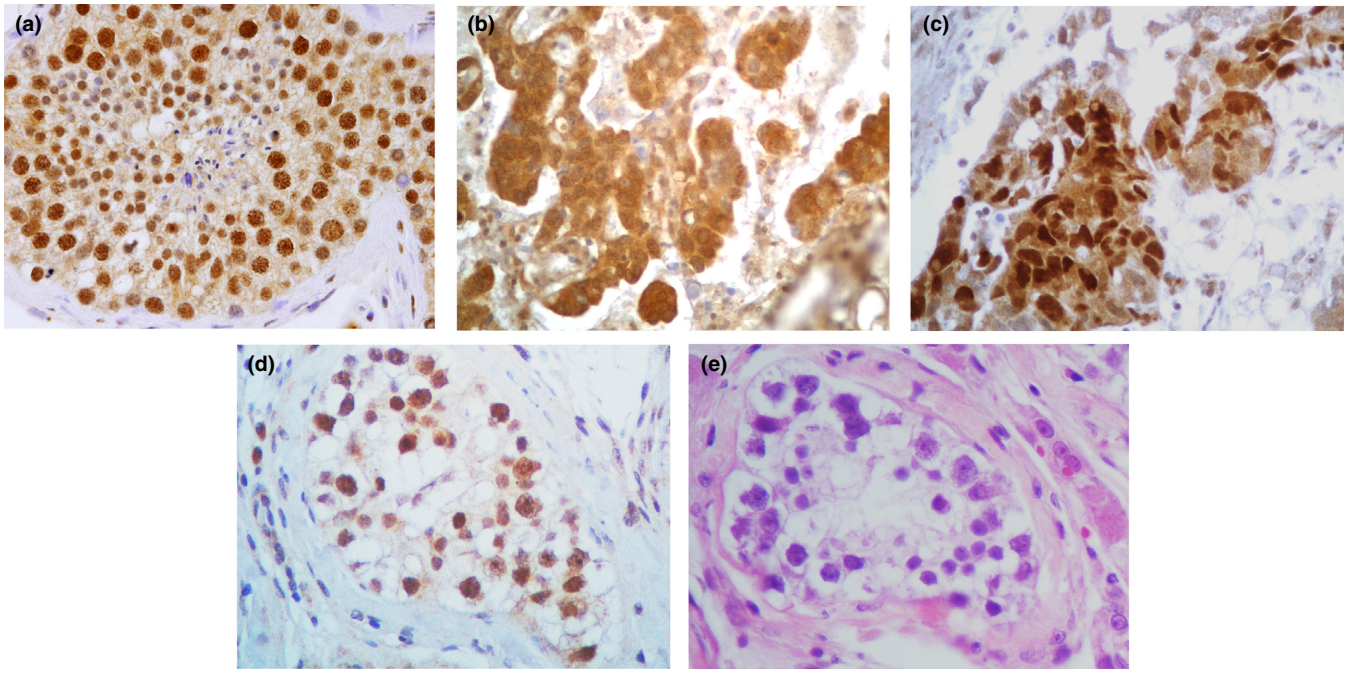
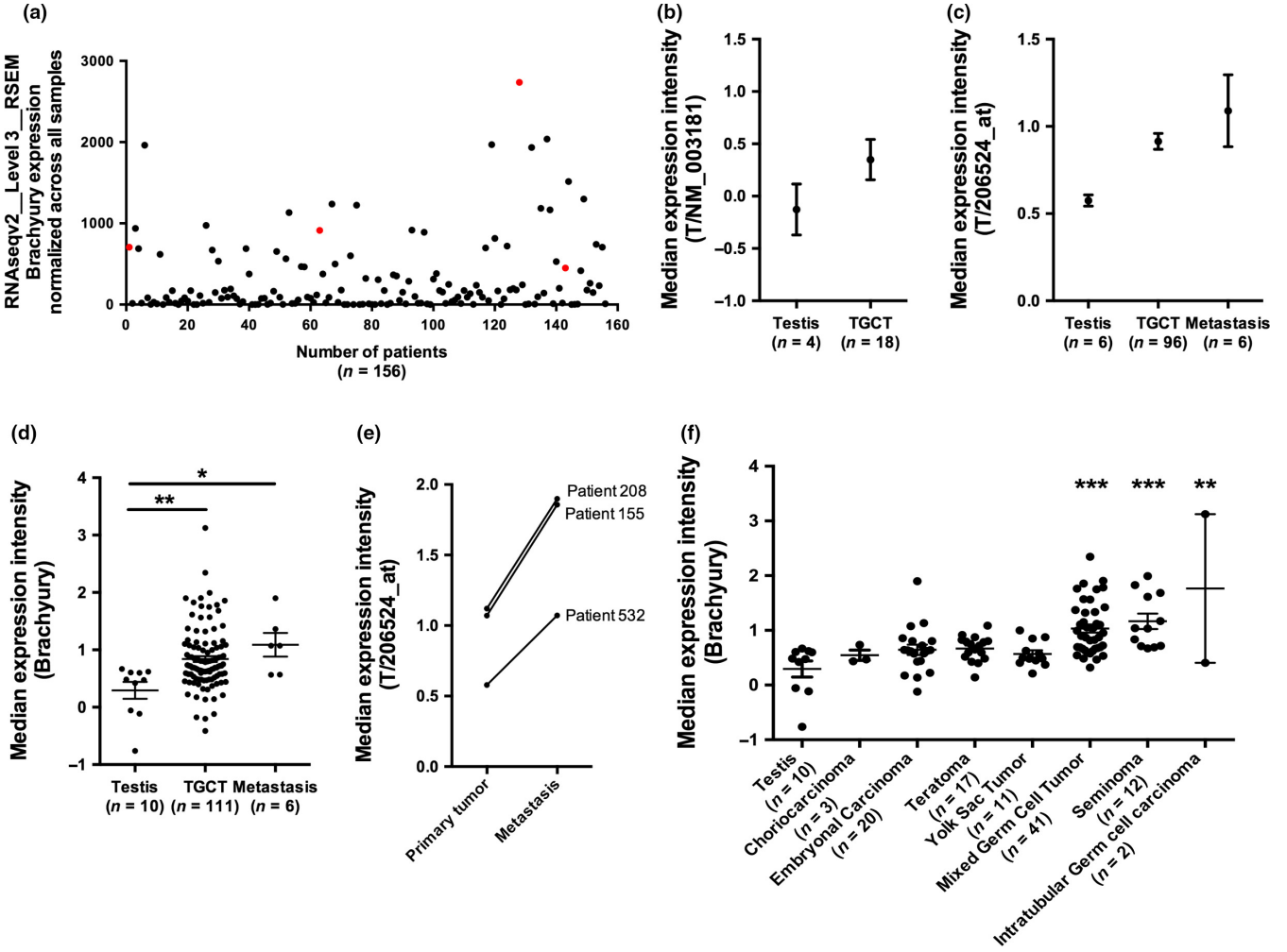


Figure 2 *Brachyury* overexpression in TGCT and metastasis. (a) *Brachyury* expression analysis from TCGA database ($n = 156$). Red dots represent the deceased patients. Expression data represent Level 3 normalization from TCGA portal (expression values were normalized across all samples). (c–d) *Brachyury* is overexpressed in TGCT and metastatic samples compared with normal testis. Data extracted from (b) Skotheim ($n = 22$) and (c) Korkola ($n = 110$) datasets. (d) Graphical representation of Korkola and Skotheim datasets together ($n = 127$) showing a statistically significant *Brachyury* overexpression in primary TGCT and metastasis. (e) Data from three patients (from Korkola dataset) with both primary and metastasis samples showed an increased expression of *Brachyury* in aggressive metastatic tissue compared with primary TGCT. (f) *Brachyury* is associated with seminoma, mixed, and intratubular subtypes. Data extracted from Oncomine database from Korkola and Skotheim datasets. TGCT, testicular germ cell tumor.



power, we have joined the two datasets (testis: $n = 10$; TGCT: $n = 111$; metastasis: $n = 6$). As observed in Fig. 2d, *Brachyury* was statistically upregulated in both TGCTs ($p < 0.01$) and metastasis ($p < 0.05$) samples. *Brachyury* upregulation was also observed in three TGCT patients with expression data for both primary and metastasis samples (Fig. 2e). Moreover, we found that Brachyury is statistically overexpressed in seminoma ($p < 0.001$), mixed ($p < 0.001$), and intratubular ($p < 0.01$) germ cell tumor subtypes (Fig. 2f).

Brachyury nuclear subcellular localization is associated with poor survival in intermediate/high-risk TGCT

The scarce clinical information from the TCGA database indicates that only four TGCT patients died, in which showed relative high levels of *Brachyury* expression (red dots in Fig. 2a). This information prompts us to explore the prognostic role of this T-box in our series of TGCT.

Regarding 2-year and 5-year event-free survival (EFS) and overall survival (OS) and clinicopathological features (Table S2), we found that lower EFS and OS were significantly associated with serum markers (AJCC) ($p < 0.001$ and $p = 0.02$), tumor stage (AJCC) ($p < 0.001$ and $p = 0.04$), number of metastasis ($p < 0.001$ and $p = 0.001$), chemotherapy response ($p < 0.001$ and $p < 0.001$), and IGCCCG risk ($p < 0.001$ and $p < 0.001$). No association was found with age, histology or Brachyury staining, and its subcellular localization (Table S2). TGCT survival is highly influenced by several factors as represented in Table S2, including the IGCCCG risk, which also is associated with other parameters (as tumor stage, metastasis, and serum markers). Moreover, the intermediate- and high-risk TGCTs are more probably resistant to therapy and urgently need novel prognostic biomarkers in this specific subgroup of TGCT patients. Then, we analyzed the prognostic impact of Brachyury expression and compartmentalization in the intermediate/high-risk TGCT group (Tables 2 and 3). We observed that staining of Brachyury independently of its subcellular localization was not associated with poor survival (Table 2). However, Brachyury positivity in the

Table 2 Survival of intermediate- or high-risk-TGCT patients according to clinical features and Brachyury staining

Clinical features	Patients *n*	Event-free survival (%)			Overall survival (%)		
		2 years	5 years	*p*-value†	2 years	5 years	*p*-value†
TGCT	31	32.3	27.6		66.5	53.7	
Age (years)							
<28.9	20	32.5	26.0	0.84	64.3	50.5	0.64
≥28.9	11	31.8	31.8		70.1	58.4	
Histology group							
Non-seminoma	26	34.9	29.9	0.68	67.5	57.3	0.44
Seminoma	5	20.0	20.0		60.0	40.0	
Histology							
Seminoma	5	20.0	20.0	0.18	60.0	40.0	0.11
Mixed tumor	8	37.5	37.5		50.0	50.0	
Mixed tumor plus teratoma	7	28.6	0.0		64.3	64.3	
Embryonal carcinoma	4	75.0	75.0		100.0	100.0	
Yolk sac tumor	4	0.0	0.0		66.7	0.0	
Immature teratoma	2	0.0	0.0		100.0	100.0	
Choriocarcinoma	1	0.0	0.0		0.0	0.0	
Mature teratoma	0	n/a	n/a		n/a	n/a	
Serum markers (AJCC)							
S0	0	n/a	n/a	0.001	n/a	n/a	0.56
S1	3	0.0	0.0		66.7	66.7	
S2	13	46.2	36.9		68.4	59.8	
S3	5	0.0	0.0		40.0	40.0	
SX	10	35.0	35.0		78.8	50.6	
Chemosensitivity							
Responsive	17	62.6	53.6	<0.001	69.5	69.5	0.17
Refractory	14	0.0	0.0		63.5	31.7	
Brachyury staining							
Negative	3	33.3	33.3	0.95	66.7	66.7	0.81
Positive	28	32.1	26.8		66.3	52.3	
Brachyury Nuclear staining only							
Negative	27	37.3	31.9	0.04	73.5	62.9	0.01
Positive	4	0.0	0.0		25.0	0.0	
Brachyury subcellular localization							
Negative	3	33.3	33.3	0.12	66.7	66.7	0.04
Only cytoplasm	24	37.8	31.5		74.2	62.3	
Nucleus (± cytoplasm)	4	0.0	0.0		25.0	0.0	

†Log-rank test; n/a – Not applicable.

nucleus was statistically associated with poor EFS and OS at 2 years and 5 years in intermediate/high-risk TGCT patients ($p = 0.04$ and $p = 0.01$, respectively) (Table 2). As shown in Fig. 3a, Brachyury nuclear-negative cases present a better median EFS compared to Brachyury nuclear-positive cases (18.2 vs. 5.9 months, $p = 0.04$). The same was shown for median OS (90.4 vs. 9.8 months, $p = 0.01$) with median follow-up of 39.2 months (Fig. 3b). Finally, the multivariate analysis showed the same tendency, however, did not reach statistical significance (HR: 3.56, $p = 0.06$) (Table 3).

In a previous study of the same series, our group showed that methylation of *MGMT* and CALCA genes was associated TGCT poor prognosis. The association of Brachyury expression and MGMT and CALCA methylation status did not find any correlation neither in overall TGCT cases ($n = 96$, $p = 0.53$) nor on the intermediate/high-risk group ($n = 31$, $p = 0.47$).

DISCUSSION

Testicular germ cell tumors (TGCT) achieved high cure rates with platinum-based regimens, yet there is a subset of refractory patients (IGCCCG, 1997; Gilbert *et al.*, 2009, Popovic *et al.*, 2015). The tumor markers (alpha fetoprotein and human chorionic gonadotrophin) decline rates after 3-week first-line chemotherapy have been used to predict clinical outcomes in high-risk TGCT and dose-dense chemotherapy for unfavorable decline seems to improve survival, although about 40% present a 2-year disease progression (Fizazi *et al.*, 2004, 2014). Besides, histology, primary tumor location, response to treatment, progression-free interval after first-line treatment, as well as levels of tumor markers, and the presence of liver, bone, or brain metastases at salvage protocols have been used as prognostic variables. Nevertheless, even in the very low-risk group, about 20% may present a 2-year disease progression (International Prognostic Factors Study *et al.*, 2010). Therefore, the high-risk TGCT claims for a biomarker able to guide better therapy with as low toxicity as possible.

Herein, we proposed, for the first time, the use of Brachyury nuclear staining as a poor prognostic biomarker for intermediate/high-risk TGCT patients. All patients with positive Brachyury nuclear staining and intermediate/high-risk according to the IGCCCG evolved with disease progression before 2 years and died before 3 years. Our *in silico* analysis is in agreement with these results, where four TGCT patients died and showed relative high levels of Brachyury expression. Brachyury nuclear positivity has been described mainly in embryonal carcinoma (74%) and seminoma (45%), and we found 18.2% and 24% in our setting, respectively (Miettinen *et al.*, 2015). In our cohort, the mixed tumors were the most common histology subtype and we were not able to segregate them for comparison

Table 3 Univariate and multivariate overall survival analysis of intermediate- or high-risk-TGCT patients by Cox model

Variable	Univariate analysis			Multivariate analysis		
	Hazard ratio	95% CI	*p*-value	Hazard ratio	95% CI	*p*-value
Age (years)						
<28.9	1	*				
≥28.9	0.75	0.23–2.46	0.64			
Histology group						
Non-seminoma	0.60	0.16–2.22	0.44			
Seminoma	1	*				
Serum markers (AJCC)						
S1	0.48	0.05–4.70	0.53			
S2	0.38	0.09–1.66	0.20			
S3	1	*				
SX	0.40	0.08–1.82	0.23			
Chemosensitivity						
Responsive	1	*		1	*	
Refractory	2.15	0.70–6.63	0.180	1.58	0.46–5.34	0.45
Brachyury Nuclear staining only						
Negative	1	*		1	*	
Positive	4.30	1.25–15.14	0.02	3.56	0.93–13.55	0.06

95% CI – 95% confidence interval. *, multivariate analysis.

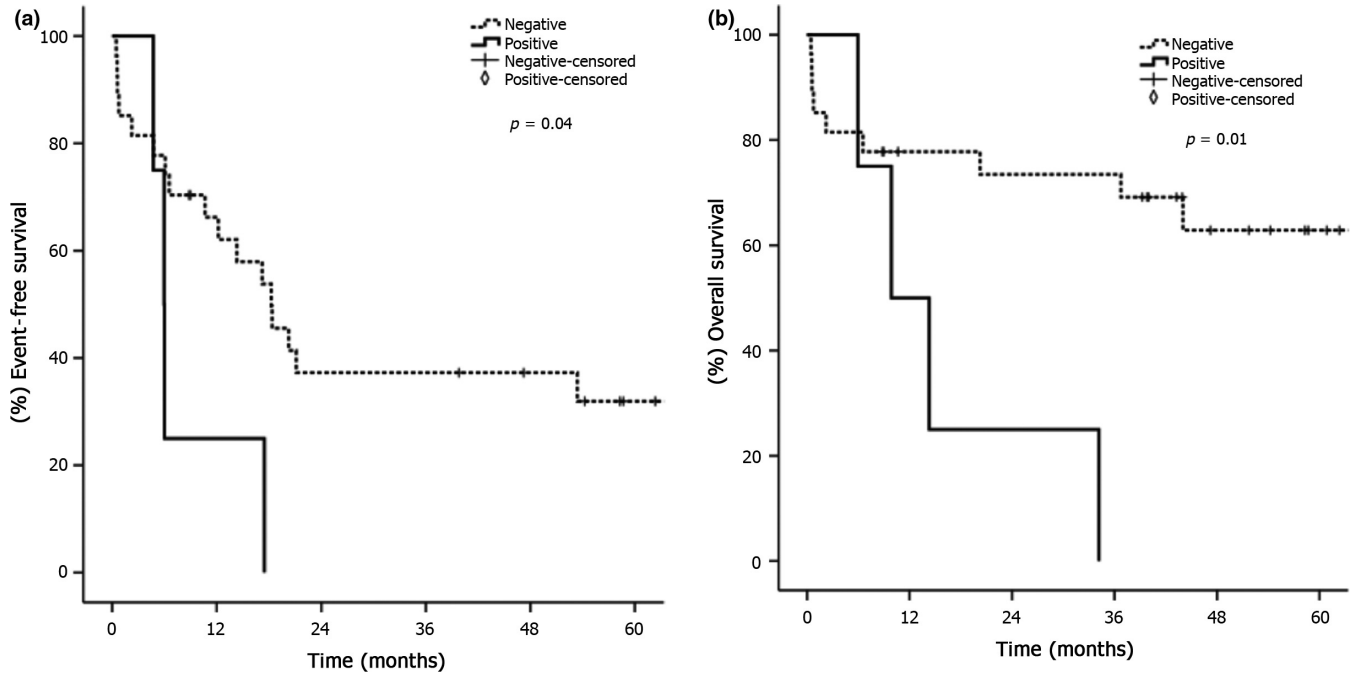
analysis, which prelude any kind of associations with tumor histology ($p = 0.91$). However, by *in silico* analysis, we found that high levels of *Brachyury* are associated with seminoma, mixed, and intratubular germ cell subtypes. We found intratubular GCNIS in 23 cases, and nuclear Brachyury was positive in 78.3%, although there was no association of nuclear Brachyury staining between GCNIS and germ cell tumors. The hypothesis of Brachyury as an early TGCT tumorigenic event of should be tested, but it seems that Brachyury remains as an important proliferative drive during TGCT tumor progression. Unfortunately, peritumoral seminiferous tubules were underrepresented in our cohort hampering any conclusion on this topic.

Our results are in accordance with the findings in other tumor types, such as GIST and prostate cancer, in which we demonstrated the association of Brachyury overexpression with tumor aggressiveness and presence of metastasis (Pinto *et al.*, 2014, 2016a,b). In primary lung carcinoma, Brachyury mRNA expression was a significant predictor of poor prognosis for 5-year disease-free survival and overall survival (Haro *et al.*, 2013). Brachyury mRNA and protein expression was also analyzed in human breast carcinomas, and it was associated with tumor recurrence and distant metastasis (Palena *et al.*, 2014). These data corroborate with the upregulation of Brachyury observed in more advanced TGCT according to our *in silico* analysis.

The impact of Brachyury in the cytoplasm has been described in several tumor types, including colorectal cancer (Kilic *et al.*, 2011), prostate cancer (Pinto *et al.*, 2014), and GIST (Pinto *et al.*, 2016a,b), and to be associated with poor prognosis. In our series of TGCT, we did not find any association with the clinical–pathological data. Previously, we have hypothesized that Brachyury protein in the cytoplasm can i) interact with cofactors to regulate oncogenic pathways, as described to other transcription factors (Green & Kroemer, 2009; Lau & Ronai, 2012); or ii) be a consequence of a dynamic process of protein shuttling between cytosol and nucleus. In this particular case, we hypothesize that Brachyury is accumulated in the cytosol of cancer cells as a consequence of enhanced translation mechanisms. As so, Brachyury presence in the cytosol can be a surrogate marker of TGCT progression. Clearly, there is much more to fully understand the role of Brachyury in the cytoplasm and further studies are warranted.

In our cohort of TGCT patients, we recently reported that well-known hallmarks of cancer, as microsatellite instability (MSI), *V600E BRAF*, and TERT promoter mutations, are not present

Figure 3 Brachyury is a novel biomarker of poor prognosis in intermediate/high-risk TGCT. Kaplan–Meier analysis of event-free (a) and overall survival (b) according to Brachyury nuclear staining. Patients with Brachyury nuclear staining present worse survival probabilities compared with nuclear-negative patients.



(Carcano *et al.*, 2016a,b). Recently, Bagrodia *et al.* (2016) found *TP53* pathway alterations associated with poor risk according to IGCCCG, especially by amplification of *MDM2*, an E3 ubiquitin-protein ligase that negatively regulates TP53. Further, *TP53* and *MDM2* alterations were associated with worse outcomes, independent of the IGCCCG model, in the same study. Moreover, our group has described *MGMT* and *CALCA* promoter methylation as associated with poor prognosis in TGCT (Martinelli *et al.*, 2016). New MDM2 inhibitors have been developed (Zhao *et al.*, 2015), as well as a second generation of DNA methylation inhibitor (Albany *et al.*, 2017). Together with anti-Brachyury vaccine (GI-6301), those new drugs open a new window of possibilities to treat the high-risk TGCT with targetable functional molecules.

Our cohort of patients is representative of TGCT, and well-known prognostic factors, such as serum markers, stage, chemosensitivity, number of metastasis sites, and IGCCCG model, were statistically significant. However, the results from intermediate/high-risk setting have limited inference due to small exploratory sample ($n = 31$). Moreover, the confidence interval of Brachyury nuclear staining in multivariate analysis is rather large (95% CI: 0.93–13.55) and the number of events is low to achieve confident and meaningful conclusions, despite the hazard ratio significant values. Therefore, further studies with a higher number of patients and with a broader representation of the distinct histological and clinical subtypes are needed to consolidate the prognostic role of Brachyury in TGCT. In conclusion, these results indicate that Brachyury plays an oncogenic role in TGCTs and its subcellular nuclear localization constitutes a novel biomarker of poor prognosis and a putative target for therapy in intermediate/high-risk TGCT patients.

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ETHICS APPROVAL

The study was conducted following the national and institutional ethical policies and was approved by the Barretos Cancer Hospital Ethical Committee (protocol 676/2013). The ethical committee classified this study as having minimal risk, ensuring confidentiality, and not resulting in any clinical influences due to changes in treatment or genetic counseling for the participants and their families. For these reasons, the ethical committee waived the need for consent.

COMPETING INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

FP and FMC were involved in acquisition of data, analysis and interpretation of data, and drafting and revising the manuscript. ECAS and CS contributed to immunohistochemistry reading and interpretation. DOV worked with data acquisition, analysis, and interpretation, as well as manuscript revision. LFL and CS made substantial contributions to manuscript revision. RMR contributed to conception and design, analysis and interpretation of data, as well as revising the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1 Correlation between Brachyury and Brachyury nuclear staining only with clinicopathological features in TGCT.
Table S2 Survival of TGCT patients according to clinical features and Brachyury staining.