Brachyury, a vaccine target, is overexpressed in triple-negative breast cancer

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Abstract

Patients diagnosed with triple-negative breast cancer (TNBC) have a high rate of tumor metastasis and a poor prognosis. The treatment option for these patients is currently chemotherapy, which results in very low response rates. Strategies that exploit the immune system for the treatment of cancer have now shown the ability to improve survival in several tumor types. Identifying potential targets for immune therapeutic interventions is an important step in developing novel treatments for TNBC. In this study, *in silico* analysis of publicly available datasets and immunohistochemical analysis of primary and metastatic tumor biopsies from TNBC patients were conducted to evaluate the expression of the transcription factor brachyury, which is a driver of tumor metastasis and resistance and a target for cancer vaccine approaches. Analysis of breast cancer datasets demonstrated a predominant expression of brachyury mRNA in TNBC and in basal vs luminal or HER2 molecular breast cancer subtypes. At the protein level, variable levels of brachyury expression were detected both in primary and metastatic TNBC lesions. A strong association was observed between nuclear brachyury protein expression and the stage of disease, with nuclear brachyury being more predominant in metastatic vs primary tumors. Survival analysis also demonstrated an association between high levels of brachyury in the primary tumor and poor prognosis. Two brachyury-targeting cancer vaccines are currently undergoing clinical evaluation; the data presented here provide rationale for using brachyury-targeting immunotherapy approaches for the treatment of TNBC.

Key Words

- ▶ brachyury
- ▶ TNBC
- ▶ tumor antigen
- ▶ vaccine target

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Introduction

Triple-negative breast cancer (TNBC), which accounts for approximately 10–15% of all breast tumors, is an aggressive breast cancer subtype defined through biomarker analysis

by the absence of estrogen (ER) and progesterone (PR) receptor expression and lack of epidermal growth factor receptor-2 (HER2) overexpression and/or amplification.

Due to the absence of expression of these receptors, no effective therapy is currently available for TNBC patients, which typically present a high rate of tumor relapse compared with patients with other subtypes of breast cancer (Carey *et al.* 2006, Haffty *et al.* 2006, Dent *et al.* 2007, O'Brien *et al.* 2010). With the recent use of genome-wide expression analysis, breast cancer has also been classified into various molecular subtypes. The initial classification, currently known as the 'intrinsic subtypes' and defined by the PAM50 assay, includes the luminal A, luminal B, HER2-enriched and basal-like groups (Perou *et al.* 2000, Prat *et al.* 2012). Tumors of the basal-like category are usually negative for ER, PR and HER2, and thus overlap with the biomarker-defined TNBC group (Metzger-Filho *et al.* 2012). Several reports have now demonstrated that these tumors frequently exhibit features of tumor-initiating cells (also designated as cancer stem cells), upregulation of mesenchymal markers and downregulation of epithelial markers, thus suggesting a possible link between the phenomenon of epithelial-mesenchymal transition (EMT) and TNBC (Gupta & Massague 2006, Sarrio *et al.* 2008, Jeong *et al.* 2012). EMT has been associated with a more invasive or metastatic tumor behavior and with the acquisition of resistance to a variety of anticancer therapies, including chemotherapy, radiation, small molecule-targeted therapies and immunotherapy (Thiery & Sleeman 2006, Creighton *et al.* 2009, Kalluri & Weinberg 2009, Huang *et al.* 2013, Larocca *et al.* 2013, Hamilton *et al.* 2014, David *et al.* 2016, Fernando *et al.* 2016).

Brachyury (encoded by the gene *T*) (Kispert *et al.* 1995) is a transcription factor that induces EMT in human carcinoma cells (Fernando *et al.* 2010) and whose expression has been reported to associate with poor prognosis in several human cancers including prostate (Pinto *et al.* 2014), lung (Haro *et al.* 2013), colon (Kilic *et al.* 2011) and breast (Palena *et al.* 2014b, Shao *et al.* 2015) among others. Our group has previously reported on an increased proportion of brachyury-expressing breast carcinomas negative for ER and PR (Palena *et al.* 2014b). However, a limited sample number precluded the evaluation of brachyury in the TNBC subgroup. In this study, the expression of brachyury was analyzed in primary and metastatic TNBC tumors using both *in silico* and immunohistochemical analyses. Increased frequency of samples positive for brachyury mRNA expression was observed in TNBC compared with other breast cancer subtypes. Furthermore, brachyury mRNA expression levels inversely correlated with the levels of

mRNA encoding for ER- α (ESR1) and PR (PGR) receptors. Immunohistochemical analysis of primary and metastatic TNBC biopsies also demonstrated variable levels of brachyury protein expression in primary and metastatic TNBC lesions. Furthermore, a strong association was observed between nuclear brachyury expression and stage of disease, with nuclear brachyury also being predominant in metastatic vs primary TNBC tumors. An association was also observed between high levels of brachyury in primary tumors and poor prognosis.

There are currently two brachyury-targeting cancer vaccines undergoing clinical evaluation (Heery *et al.* 2015b) (www.clinicaltrials.gov/ct2/show/NCT02179515). The data presented here provide a rationale for using brachyury-targeting immunotherapy approaches for the treatment of TNBC.

Methods

Tumor cell lines

Tumor cell lines were purchased from the American Tissue Culture Collection (ATCC), which uses STR analysis for identity verification. Cells were grown in DMEM medium (Corning) supplemented with 10% fetal bovine serum (Gemini Bio-Products), 1X antibiotic/antimycotic solution (Corning) and 4 μ g/mL recombinant human insulin (Gibco). Cell lines were used for experiments at the following passages from purchased stocks: MDA-MB-231, passage 6; MDA-MB-436, passage 4; Hs 578T, passage 6; BT-474, passage 2; ZR-75-1, passage 4; and MCF7, passage 1.

Patients and tissue collection

Seventy-seven patients with histologically diagnosed primary TNBC and ten patients with metastatic TNBC were enrolled in the Inter-Institutional Multidisciplinary BioBank of the Biomarker Discovery and Advanced Technologies (BioDAT) Laboratory, IRCCS San Raffaele Pisana, Rome, Italy. In collaboration with the Surgical and Pathology Departments of San Giovanni Addolorata Hospital and the Medical Oncology Unit of the 'Tor Vergata' Clinical Center, Rome, Italy, primary breast and metastatic tumor tissue samples were collected at the time of surgery. Patient characteristics are provided in Table 1. Clinical follow-up data were available from 58 of 77 patients with primary TNBC. Patients received the following treatment regimens: 30 of 58 received anthracycline-based therapy; 4 of 58 received

Table 1 Patient characteristics.

	Overall population (n=87)	Follow-up population (n=58)
Primary breast cancer (n=77)		
Age, year (Mean±s.d.)	63±16	63±16
Menopausal status		
Pre	27 (35%)	21 (36%)
Post	50 (65%)	37 (64%)
Pathological diagnosis		
Infiltrating ductal carcinoma	70 (91%)	54 (93%)
Infiltrating lobular carcinoma	5 (6%)	2 (3.5%)
Others ^a	2 (3%)	2 (3.5%)
Grading		
1	0 (0%)	0 (0%)
2	0 (0%)	0 (0%)
3	77 (100%)	58 (100%)
Stage		
I	28 (36%)	21 (36%)
II	27 (35%)	24 (42%)
III	20 (26%)	13 (22%)
IV	2 (3%)	0 (0%)
p53 expression		
Negative	41 (53%)	26 (45%)
Positive	36 (47%)	32 (55%)
Ki67 expression		
Median (range)	70 (40–90)	56 (40–90)
Positive, n (%) ^b	76 (99%)	57 (98%)
Metastatic breast cancer (n=10)		
Age, year (Mean±s.d.)	63±17	
Menopausal status		
Pre	4 (40%)	
Post	6 (60%)	
Pathological diagnosis		
Infiltrating ductal carcinoma	9 (90%)	
Infiltrating lobular carcinoma	1 (10%)	
p53 expression		
Negative	8 (80%)	
Positive	2 (20%)	
Ki67 expression		
Median (range)	63 (50–80)	
Positive, n (%) ^b	9 (90%)	

^aIncludes a case of comedocarcinoma and a metaplastic breast cancer;

^bKi67 positivity defined as ≥20.

non-anthracycline-based regimens (CMF); 5 of 58 received adjuvant radiotherapy alone; 8 of 58 did not receive treatment and treatment data were not available for the remaining 11 of 58 patients. Informed consent was obtained from each participating subject. The study was performed under the appropriate institutional ethics

approval and in accordance with the principles embodied in the Declaration of Helsinki.

Immunohistochemical detection of brachyury

Sections of formalin-fixed, paraffin-embedded tissues were evaluated for brachyury expression by using a rabbit monoclonal anti-brachyury antibody (MAb 54-1) at a 1:500 dilution (Hamilton et al. 2015). Staining was performed on the Ventana BenchMark XT automated staining platform with the UltraView Universal DAB Detection Kit (Roche) according to manufacturer's instructions. Two pathologists independently evaluated the tumor and normal tissue samples in a blinded, randomized manner. For each slide, three to five random fields were evaluated; for each field, the percentage of positive tumor cells was calculated as: ((number of positive tumor cells/total number of tumor cells)×100). Nuclear, cytoplasmic and total brachyury staining was independently scored, with brachyury being observed either in the nucleus, the cytosol or both compartments of the tumor cells. For calculation of total brachyury expression (Table 2), the percentage of tumor cells with mutually exclusive cytosolic or nuclear staining was added. Tumor cells showing brachyury in both compartments were scored only once. The relative staining intensity was scored as weak (+) for pale brown intensity, moderate (++) for intermediate brown intensity and strong (+++) for intense, dark brown immunoprecipitate. Immunoreactivity index was calculated by multiplying the percentage of positive cells by the staining intensity. For normal tissues, the percentage of reactivity was individually evaluated for each cell type and calculated as: ((number of positive cells/total number of cells of the same type)×100).

In silico analysis of the TCGA dataset

Relative expression levels of indicated mRNAs were assessed using the TCGA dataset containing data from 1026 breast carcinoma patients (<http://cancergenome.nih.gov>). For the analysis, breast cancer samples were subdivided into three groups according to the level of brachyury (T) expression: 881 of 1026 samples with no detectable brachyury expression were classified as negative (neg). The remaining 145 samples were ranked and subdivided into 'brachyury-high' (high, 73 of 1026) and 'brachyury-low' (low, 72 of 1026) groups based on an arbitrary cutoff set at the median value for the 145 samples. The level of expression of mRNA encoding

Table 2 Immunohistochemical detection of brachyury in biopsies obtained from primary lesions of TNBC patients.

Pt	Stage	Ki67	p53	Nuclear brachyury			Cytoplasmic brachyury			Total brachyury		
				Pos (%)	SI	Index	Pos (%)	SI	Index	Pos (%)	SI	Index
1	I	90	NEG	100	+++	300	70	++	140	100	+++	300
2	I	25	POS	100	+++	300	100	++	200	100	+++	300
3	I	35	POS	70	+++	210	100	++	200	100	+++	300
4	I	60	POS	60	+++	180	80	++	160	90	+++	270
5	I	80	NEG	90	+++	270	10	+	10	90	+++	270
6	I	60	NEG	20	++	40	80	+++	240	80	+++	240
7	I	80	NEG	80	++	160	50	+	50	80	++	160
8	I	90	POS	20	+	20	80	+	80	80	+	80
9	I	90	POS	30	+	30	80	++	160	80	++	160
10	I	40	NEG	70	++	140	10	+	10	70	++	140
11	I	70	POS	50	+++	150	70	+	70	70	+++	210
12	I	20	POS	40	+++	120	60	++	120	70	+++	210
13	I	70	NEG	20	+	20	70	++	140	70	++	140
14	I	20	NEG	70	+++	210	70	+	70	70	+++	210
15	I	35	POS	60	+++	180	20	+	20	60	+++	180
16	I	40	POS	60	++	120	40	+	40	60	++	120
17	I	60	POS	20	+	20	50	+	50	50	+	50
18	I	80	NEG	20	++	40	50	+	50	50	++	100
19	I	90	POS	40	++	80	40	+	40	40	++	80
20	I	50	NEG	40	++	80	10	+	10	40	++	80
21	I	25	NEG	10	+	10	40	+	40	40	+	40
22	I	40	NEG	25	++	50	10	+	10	30	+	60
23	I	40	NEG	15	++	30	20	+	20	25	+	50
24	I	70	POS	15	+	15	15	+	15	20	+	20
25	I	80	POS	20	+	20	10	+	10	20	+	20
26	I	40	POS	20	++	40	0	–	0	20	++	40
27	I	70	NEG	10	++	20	10	+	10	15	++	30
28	I	90	POS	0	–	0	0	–	0	0	–	0
29	IIA	25	POS	100	++	200	10	+	10	100	++	200
30	IIA	90	POS	80	+++	240	80	++	160	100	+++	300
31	IIA	60	POS	20	+++	60	100	++	200	100	+++	300
32	IIA	90	POS	80	+++	240	100	+++	300	100	+++	300
33	IIA	40	POS	100	+++	300	10	++	20	100	+++	300
34	IIA	45	POS	30	+	30	100	++	200	100	++	200
35	IIA	85	POS	90	+++	270	20	+	20	90	+++	270
36	IIA	90	POS	80	+++	240	60	+	60	90	+++	270
37	IIA	90	NEG	70	+++	210	80	+	80	80	+++	240
38	IIA	25	NEG	80	+++	240	10	+	10	80	+++	240
39	IIA	70	POS	30	++	60	80	+	80	80	++	160
40	IIA	40	POS	80	++	160	50	++	100	80	++	160
41	IIA	90	NEG	70	+++	210	20	++	40	70	+++	210
42	IIA	35	POS	50	++	100	30	+	30	50	++	100
43	IIA	60	NEG	25	++	50	10	+	10	30	++	60
44	IIA	70	POS	10	+	10	0	–	0	10	+	10
45	IIA	60	POS	10	+	10	0	–	0	10	+	10
46	IIA	90	NEG	10	+	10	0	–	0	10	+	10
47	IIA	25	NEG	5	+	5	0	–	0	5	+	5
48	IIB	35	POS	75	++	150	100	+	100	100	++	200
49	IIB	20	POS	20	++	40	80	+	80	80	+	160
50	IIB	60	NEG	40	+++	120	70	++	140	70	+++	210
51	IIB	80	NEG	70	+++	210	30	+	30	70	+++	210
52	IIB	90	NEG	50	++	100	50	+	50	60	++	120
53	IIB	80	NEG	60	++	120	20	+	20	60	++	120
54	IIB	90	POS	30	+++	90	20	++	40	40	+++	120
55	IIB	90	NEG	30	+	30	30	+	30	30	+	30
56	IIIA	90	POS	80	+++	240	100	+	100	100	+++	300

(Continued)

Table 2 Continued.

Pt	Stage	Ki67	p53	Nuclear brachyury			Cytoplasmic brachyury			Total brachyury		
				Pos (%)	SI	Index	Pos (%)	SI	Index	Pos (%)	SI	Index
57	IIIA	90	NEG	100	+++	300	0	–	0	100	+++	300
58	IIIA	10	NEG	90	+++	270	50	++	100	90	+++	270
59	IIIA	30	NEG	90	+	90	30	+	30	90	+	90
60	IIIA	60	NEG	80	++	160	60	++	120	90	++	180
61	IIIA	80	NEG	70	+++	210	60	+	60	80	+++	240
62	IIIA	70	NEG	70	+++	210	10	+	10	70	+++	210
63	IIIA	70	NEG	40	+	40	60	+	60	60	+	60
64	IIIA	50	NEG	50	++	100	10	+	10	50	++	100
65	IIIA	60	NEG	40	+	40	15	+	15	45	+	45
66	IIIB	95	POS	40	+++	120	100	+	100	100	++	200
67	IIIB	35	NEG	100	+++	300	50	++	100	100	+++	300
68	IIIB	70	NEG	100	+++	300	30	+	30	100	++	200
69	IIIB	80	NEG	100	+++	300	10	+	10	100	+++	300
70	IIIB	80	NEG	90	+++	270	20	+	20	90	+++	270
71	IIIB	40	POS	80	+++	240	40	+	40	80	+++	240
72	IIIB	50	POS	60	++	120	60	++	120	80	++	160
73	IIIB	80	NEG	70	++	210	70	+	70	80	+++	240
74	IIIB	90	NEG	50	+++	150	50	+	50	50	+++	150
75	IIIB	90	POS	30	+	30	30	+	30	40	+	40
76	IV	90	NEG	30	++	60	40	+	40	50	++	100
77	IV	80	NEG	15	+++	45	10	+	10	10	+++	30

NEG, negative; POS, positive; SI, staining intensity.

ER- α (ESR1), ER- β (ESR2), PR (PGR) and HER2 (ERBB2) were evaluated in each group. A subset of tumors in the database ($n=513$) for which data were available regarding the four intrinsic subtypes using the PAM50 discriminator assay (Parker *et al.* 2009) was further assessed for brachyury expression (Cancer Genome Atlas Network 2012). Samples were also subdivided into subgroups according to ER, PR and HER2 status for comparison of the levels of mRNAs encoding for brachyury, various EMT transcription factors and the chemokine IL8. Samples were classified as triple positive (ER+, PR+ and HER2+), TNBC (ER–, PR– and HER2–) and non-TNBC. Non-TNBC corresponds to tumors that were at least positive for one marker (ER, PR or HER2). All data were analyzed using the Nexus Expression 3 analysis software package (BioDiscovery).

Immunofluorescence

Tumor cells were cultured in 96-well black, clear-bottom plates (Greiner Bio-One). After fixation with 3% paraformaldehyde (Electron Microscopy sciences), permeabilization with 0.05% Triton X and blockade by 1X PBS supplemented with 1% BSA and 10% goat serum, cells were stained using antibodies reactive against

vimentin (Dako), fibronectin, ZO1 (BD Biosciences, San Jose, CA, USA), and brachyury (Mab 54-1) and Alexa Fluor 488 anti-mouse or anti-rabbit secondary antibodies. Nuclei were stained using DAPI (Thermo Fisher Scientific), and images were acquired using a Celigo S Cell Imaging Cytometer (Nexcelom Bioscience, Lawrence, MA, USA). To silence brachyury expression, control and brachyury-targeting ON-TARGETplus SMARTpool siRNAs were purchased from Dharmacon and used according to the manufacturer's instructions (GE Life Sciences, Pittsburgh, PA, USA). Cells were incubated for 72h in an antibiotic-free medium before it is used in the analysis of various markers by immunofluorescence.

Quantitative real-time PCR

Total RNA was prepared using the PureLink RNA Mini Kit (Thermo Fisher Scientific) and reverse-transcribed with the XLAScript cDNA MasterMix (WordWide Life Sciences). The resulting cDNA (10ng) was amplified in triplicate with the following TaqMan human gene expression assays (Life Technologies): *T* (brachyury) (Hs00610080_m1) and *GAPDH* (4326317E) using a 7300 Applied Biosystems instrument. Expression of brachyury relative to GAPDH was calculated as $2^{-(Ct(GAPDH) - Ct(target\ gene))}$.

Statistical methods

The unpaired two-sample Student's *t*-test was used for comparison of mean expression levels of indicated transcripts in samples in the TCGA dataset. For survival analysis, samples for which clinical follow-up was available ($n=58$) were assigned into a low vs high brachyury group, based on an arbitrary cutoff of 240 for the overall brachyury reactivity index, set at the 75th percentile for the overall population ($n=87$). A Kaplan-Meier analysis was used to evaluate the association between brachyury expression and relapse-free survival. Cox proportional hazards regression model was used to adjust for potential confounding factors such as stage

of disease and p53 expression. All statistical tests were two sided.

Results

Brachyury mRNA expression in TNBC

Samples ($n=1026$) from the TCGA breast cancer database were subdivided according to the level of brachyury (T) mRNA expression into brachyury negative ($n=881$), low ($n=72$) or high ($n=73$) groups, and subsequently analyzed for the level of expression of ER-, PR- and HER2 receptor-encoding mRNAs. The most significant associations were observed with ER- α - and PR-encoding mRNAs

Figure 1

Brachyury mRNA upregulation in TNBC and basal breast cancers. (A) Analysis of expression of indicated transcripts in the breast cancer TCGA dataset, according to the level of brachyury (T) mRNA expression. Percent of tumors in the TCGA database that are positive for brachyury (T) mRNA expression in samples classified based on their hormone receptor expression (B) or according to the molecular classification (C). Analysis of expression of indicated transcripts in breast cancer samples of the TCGA database, classified as triple positive vs TNBC, in accordance with their expression of hormone receptors (D). Statistics were calculated using an unpaired *t*-test (* $P < 0.05$; *** $P < 0.001$; **** $P < 0.0001$; ns, not significant).

(ESR1 and PGR, respectively), which demonstrated a marked decrease of expression with increasing levels of brachyury (Fig. 1A). However, this reverse association was not seen in relation to HER2- or ER- β encoding mRNAs (ERBB2 and ESR2, respectively, Fig. 1A). Further analysis of samples subdivided according to the HER2, ER and PR status demonstrated a predominant expression of brachyury mRNA among ER- vs ER+ (38 vs 6%), PR- vs PR+ (31 vs 6%), TNBC vs triple-positive (47 vs 3%) or TNBC vs non-TNBC (47 vs 10%) samples. In contrast to ER and PR, no difference was observed in the percentage of brachyury-positive tumors subdivided based on their HER2 expression (Fig. 1B). Brachyury mRNA expression was also evaluated in samples from the same dataset stratified based on the POM50 gene signature (Parker et al. 2009, Cancer Genome Atlas Network 2012). As shown in Fig. 1C, brachyury mRNA was detectable in 9 of 232 (3.9%) luminal A, 11 of 128 (8.6%) luminal B, 11 of 58 (19.0%) HER2-enriched, and 40 of 95 (42.1%) basal breast carcinoma samples.

In addition to brachyury, the TCGA dataset was used to interrogate the expression of various transcription factors known to induce EMT. Although brachyury (T) mRNA was predominantly expressed in TNBC (47 of 101) vs triple-positive (3 of 88) breast carcinoma samples, only a slight increase of SNAI1, a decrease of ZEB1 and no changes in the mRNA levels of TWIST1, SNAI2 or ZEB2 were observed in TNBC vs triple-positive breast tumors (Fig. 1D). Interestingly, we also noted a significant increase in IL8 mRNA levels in TNBC vs triple-positive breast tumors (Fig. 1E), a result in support of our previous studies demonstrating a positive association between brachyury and the IL8 axis in breast cancer (Fernando et al. 2011).

Figure 2

Immunohistochemical analysis of brachyury expression in primary and metastatic TNBC. Representative photomicrographs of brachyury staining on primary TNBC cases are reported on panels A and B. (A) Stage IIIB T2N3aM0 primary IDC (patient 71) showing positive nuclear and cytoplasmic staining in 80 and 40% of cancer cells, respectively. (B) Stage I T1cN0M0 primary IDC (patient 28) showing negative (<5%) nuclear and cytoplasmic staining in cancer cells. (C) Pleural metastatic lesion (patient 84) showing positive nuclear and cytoplasmic staining in 80 and 20% of cancer cells, respectively. (D) Detail of brachyury staining at higher magnification (400x). (E) and (F) represent bone (patient 86) and lymph node (patient 78) metastasis with positive nuclear (60 and 100%, respectively) and cytoplasmic (70 and 100%, respectively) brachyury expression in cancer cells. Magnification: 200x (except panel D: 400x).

Table 3 Immunohistochemical detection of brachyury in biopsies obtained from metastatic lesions of TNBC patients.

Pt	Site	Ki67	p53	Nuclear brachyury			Cytoplasmic brachyury			Total brachyury		
				Pos (%)	SI	Index	Pos (%)	SI	Index	Pos (%)	SI	Index
78	LN	30	NEG	100	+++	300	100	+	100	100	+++	300
79	LN	90	NEG	90	+++	270	50	+	50	100	+++	300
80	LN	65	POS	80	+++	240	0	–	0	80	+++	240
81	LN	50	NEG	70	+++	210	50	+	50	80	+++	240
82	LN	70	NEG	80	+++	240	0	–	0	80	+++	240
84	Dist	90	NEG	80	+++	240	20	+	20	80	+++	240
86	Dist	60	NEG	60	++	120	70	++	140	80	++	160
83	Dist	50	NEG	60	+++	180	40	++	80	70	+++	210
87	Dist	10	POS	60	+++	180	20	+	20	60	+++	180
85	Dist	80	NEG	30	++	60	20	+	20	40	++	80

SI: staining intensity; NEG: negative; POS: positive. LN: lymph node; Dist: distant metastasis

Brachyury protein in primary and metastatic TNBC

Brachyury protein expression was assessed by IHC in 87 tumor tissues from patients diagnosed with TNBC by using a rabbit monoclonal anti-brachyury antibody (MAb 54-1) as described previously (Hamilton et al. 2015). Among 77 primary TNBC tumors, 71 of 77 (92%) showed some level of brachyury expression in >10% of the cancer cells, either in the nucleus or the cytoplasmic compartment (Table 2). As previously reported with other types of carcinomas, expression of brachyury was highly variable among primary tumors, with the percentage of brachyury-positive cells ranging from 10 to 100% and the intensity of staining varying between (+) and (+++). Representative images of two primary TNBC cases positive vs negative for brachyury expression are shown in Fig. 2A and B, respectively. In addition to primary tumors, brachyury expression was evaluated in metastatic lesions from 10 patients with TNBC (Table 3 and representative images in Fig. 2C–F). Brachyury was observed in 100% of metastasis cases, the majority of cases showing high-intensity staining for brachyury (+++) in 40–100% of the tumor cells.

Expression of brachyury was also evaluated in breast tissues adjacent and distant to the tumor. As shown in Fig. 3, a gradient of expression was observed with the highest degree of brachyury positivity detected in the tumor > adjacent breast > distant breast tissue. In the examples shown in Fig. 3A and D, the primary tumor overall nuclear and cytoplasmic brachyury staining ranged from 20 to 80% of the cancer cells, whereas expression in breast tissues adjacent vs distant to tumor corresponded to

approximately 10% (Fig. 3B and E) and <5% (Fig. 3C and F) of the cells, respectively. Similarly, brachyury expression in the primary tumor shown in Fig. 3G corresponded to 80% of the cells, whereas expression in histologically normal breast tissue from the same patient demonstrated <1% positivity for brachyury (Fig. 3H). These results are in agreement with our previous study using a different anti-brachyury monoclonal antibody (Palena et al. 2014b), in which brachyury-positive cells were observed in the breast tissue adjacent (15 of 27 cases) but not distal to the tumor, whereas no expression was detected in 14 benign breast tissues. The findings had an exception of two fibroadenoma cases in which focal expression of brachyury was observed. At present, it is unknown whether brachyury-expressing cells in the tissues adjacent to the tumor are cancer cells that have migrated toward the surrounding stroma or correspond to normal stromal cells that upregulated brachyury in response to tumor-derived secreted factors.

In agreement with previous studies, brachyury protein was observed either in the nucleus and/or the cytosol of the TNBC cells. A comparative analysis of brachyury expression demonstrated a significant increase in nuclear brachyury protein levels, as detected by an increased reactivity index, in metastatic vs primary tumor samples ($P=0.02$, Fig. 4A), an association that was not observed with cytoplasmic brachyury. A similar association between nuclear localization of brachyury and clinical stage of disease was also observed, with samples from higher tumor stages (III–IV) having higher

Figure 3

Immunohistochemical analysis of brachyury expression in primary TNBC and normal breast tissues adjacent or distant to the tumor. (A) Stage IIB T2N1aM0 primary tumor (patient 54) showing positive nuclear and cytoplasmic staining in 30 and 20% of cancer cells, respectively; (D) Stage IIA T2N0M0 primary tumor (patient 37) showing nuclear and cytoplasmic staining in 70 and 80% of cancer cells, respectively. Panels (B) and (E) correspond to brachyury staining in breast tissues adjacent to the tumor. Panels (C) and (F) correspond to brachyury staining in breast tissues distant from the tumor. (G) Stage IIA T2N0M0 primary tumor (patient 30) showing both nuclear and cytoplasmic staining in 80% of cancer cells. (H) Brachyury staining in histologically normal breast tissue surrounding the tumor. Panel magnification: 200 $\times$.

Figure 4

Prevalence of nuclear brachyury expression in metastatic and high-grade TNBC tumors. Brachyury reactivity index observed in the nucleus or the cytoplasm of primary vs metastatic (A) or TNBC samples according to stage (B, C and D). Samples were analyzed by IHC with an anti-brachyury monoclonal antibody. *P* values were calculated using an unpaired *t*-test, with values for panel B representing a comparison between stage I and stages III–IV. (ns, not significant).

nuclear brachyury reactivity index ($P=0.012$) than samples of stage I (Fig. 4B). The same difference was observed when combined samples from tumors stages I to II were compared with those of stages III–IV in which nuclear expression of brachyury was significantly

Figure 5

Brachyury expression and prognosis. Kaplan–Meier estimates of recurrence-free survival in 58 cases of TNBC classified based on the brachyury expression level. The two groups (low vs high brachyury) were defined based on the overall brachyury reactivity index set at a cutoff of 240, corresponding to the 75th percentile of expression in the overall population ($n=87$).

higher in tumors of advanced stages, both in terms of reactivity index ($P=0.02$) and % positive nuclei ($P=0.007$, Fig. 4C). This is a correlation that was not observed with cytoplasmic brachyury expression (Fig. 4D).

Brachyury and TNBC prognosis

To evaluate a potential association of brachyury expression in primary TNBC with prognosis, samples for which clinical follow-up was available ($n=58$) were assigned into low vs high brachyury groups. It was

Table 4 Multivariate Cox proportional hazards regression analysis of the predictive value of clinical–pathological variables and brachyury expression on relapse-free survival of TNBC patients.

Variable	n	Recurrence		HR (CI)	P value
		Yes	No		
Stage of disease					
I	21	2 (10%)	19 (90%)		
II	24	4 (17%)	20 (83%)		
III	13	4 (31%)	9 (69%)	1.83 (0.60–5.57)	0.285
p53 expression					
Negative	26	4 (15%)	22 (85%)		
Positive	32	6 (19%)	26 (81%)	1.34 (0.30–5.91)	0.699
Brachyury expression ^a					
Negative (<240)	41	5 (12%)	36 (88%)		
Positive (≥240)	17	5 (29%)	12 (71%)	3.92 (1.02–15.1)	0.047

^aCategorized according to an arbitrary cutoff calculated as the 75th percentile of brachyury reactivity index for total brachyury staining in the overall population ($n=87$).

done based on an arbitrary cutoff of 240 for the overall brachyury reactivity index, set at the 75th percentile for the overall population ($n=87$). A Kaplan–Meier estimate of survival showed that high brachyury expression in the primary tumor is significantly associated with decreased survival ($P=0.03$, $n=58$, long-rank test=2.17, Fig. 5). To minimize the effect of clinical–pathological variables that might cause false-positive association between brachyury and poor prognosis, a multivariate Cox proportional hazards regression survival analysis was conducted. It was found that high brachyury expression (reactivity index ≥ 240) significantly associates with low relapse-free survival ($P=0.047$, $n=58$, COXPH, HR=3.92, CI=1.02–15.1), compared with the low brachyury expression group (Table 4).

Brachyury expression in TNBC cell lines

Immunofluorescent staining of mesenchymal vimentin and epithelial ZO1 proteins was used for phenotypic confirmation of a panel of TNBC (MDA-MB-231, MDA-MB-436 and Hs578T) and non-TNBC (BT-474, ZR-75-1 and MCF7) human breast carcinoma lines. As expected, strong expression of mesenchymal vimentin and little expression of epithelial ZO1 were observed with TNBC cells, whereas non-TNBC cells showed high amounts of ZO1 in the absence of vimentin expression (Fig. 6A). When expression of brachyury mRNA was evaluated in each cell line, higher levels were observed in TNBC vs non-TNBC cells (Fig. 6B). The potential role of brachyury in tumor EMT was also investigated with MDA-MB-436

Figure 6

Brachyury associates with mesenchymal features in human TNBC cell lines. (A) Immunofluorescent detection of vimentin and ZO1 (green signal) in a panel of TNBC and non-TNBC cell lines. (B) Relative brachyury mRNA expression levels in the various cell lines. Error bars indicate the standard error of the mean of triplicate measurements. (C) Silencing brachyury expression in MDA-MB-436 cells is associated with a reduction in the expression of mesenchymal markers vimentin and fibronectin and increased expression of the epithelial marker ZO1 (green signal). Blue signal corresponds to DAPI staining of nuclei.

cells, in which silencing of brachyury resulted in reduced expression of vimentin and fibronectin and increased expression of epithelial ZO1 (Fig. 6C). These observations thus suggested that brachyury not only associates with but is also required to maintain a mesenchymal-like phenotype in the MDA-MB-436 triple-negative breast cancer cell line.

Discussion

Elucidation of novel, potential therapeutic targets for the treatment of TNBC remains an urgent need in the absence of current, effective treatment for this aggressive tumor type. Our group and others have previously described the selective expression of the transcription factor brachyury, an immunotherapy target and a driver of tumor EMT (Palena *et al.* 2007, Fernando *et al.* 2010, Hamilton *et al.* 2013, Palena *et al.* 2014a, Palena & Hamilton 2015), in primary and metastatic lesions of invasive ductal carcinomas of the breast (Palena *et al.* 2014b, Shao *et al.* 2015). In this study, we further expanded those previous observations and demonstrated for the first time a prevalent expression of brachyury mRNA and protein in TNBC compared with other breast cancer subtypes. Variable levels of brachyury protein were observed by IHC in approximately 90% of primary and 100% of metastatic TNBC lesions analyzed. However, expression of brachyury was highly variable among primary tumors, with the percentage of positive cells ranging from 10 to 100% and the intensity of staining varying between (+) and (+++). Based on the relevant role of brachyury in tumor dissemination and/or resistance to anticancer treatments, it is expected that a small fraction of brachyury-positive cells in a tumor mass could have a major impact in terms of clinical outcome. With this functional relevance of brachyury in mind, a tissue has been designated here as 'brachyury positive' if at least 10% of the cancer cells exhibited some level of staining.

It is important to point out that in addition to providing evidence of variable levels of brachyury expression in TNBC, the data presented here also indicate that some level of brachyury could be present in normal breast tissues adjacent or distal to the tumor. These results contrast with our previous observations, in which brachyury was not detected in normal breast tissues from patients with benign conditions (Palena *et al.* 2014b). Two recently completed phase I clinical trials of a yeast-brachyury vaccine (Heery *et al.* 2015b) or a MVA-brachyury-TRICOM vaccine (Heery *et al.* 2015a) demonstrated no

evidence of any autoimmune occurrence in the presence of measurable brachyury-specific CD4+ and CD8+ T-cell responses, even in normal testis and thyroid tissues that were previously shown to express some level of the brachyury protein. However, the presence of brachyury in normal breast tissues surrounding TNBC raises concern that an autoimmune event against those tissues could take place in vaccinated patients. This warrants careful evaluation in future studies of brachyury-based vaccines.

Three cancer vaccine platforms against brachyury have been developed and two are currently undergoing phase I/II clinical evaluation (Hamilton *et al.* 2013, Gabitzsch *et al.* 2015, Heery *et al.* 2015a,b). In phase I clinical studies, brachyury-based vaccines have demonstrated the ability to elicit CD8+ and CD4+ brachyury-specific T-cell immune responses in the blood of patients after vaccination. These brachyury-specific T cells are shown to produce IFN γ , IL2 and TNF α and/or to express CD107a, a marker of lytic potential. Although some evidence of clinical response was observed, the small number of patients evaluated precluded the analysis of any potential association between the degree of antigen-specific immune response and clinical outcome. Further studies with larger number of patients need to be conducted to investigate whether such association could be established. In a previous report, we have shown that brachyury-specific T cells generated from the blood of cancer patients can lyse basal MDA-MB-231 cancer cells in an MHC-restricted manner (Palena *et al.* 2014b). Those experiments were conducted with unfractionated CD8+ T cells, and it is expected that a higher degree of lysis would be observed with tetramer-isolated, brachyury-specific T cells.

Although the molecular classification of breast cancer into various subtypes has revealed a high degree of heterogeneity in this disease, the majority of TNBC cases (56–95%) can be molecularly categorized as basal-like (Prat *et al.* 2012). Tumors of this subtype are defined by the expression of genes typically present in normal breast basal/myoepithelial cells and are characterized by a high proliferation index, p53 mutations and an aggressive course with frequent tumor relapse (Reis-Filho & Tutt 2008). The phenomenon of EMT, a phenotypic switch that allows epithelial cells to acquire motility, invasiveness and resistance to cell death while exhibiting features characteristic of mesenchymal cells, is being postulated as a relevant mechanism that fosters progression toward metastatic disease (Kalluri & Weinberg 2009). In agreement, it is the aggressive basal-like group of breast cancer that commonly exhibits mesenchymal features,

including expression of vimentin, smooth-muscle-actin (SMA) and N-cadherin in place of epithelial E-cadherin (Blick *et al.* 2008, Sarrio *et al.* 2008).

The transcription factor brachyury has been previously shown to induce phenotypic changes in carcinoma cells reminiscent of an EMT, a phenomenon associated with tumor dissemination, metastasis and acquisition of resistance to a variety of antitumor therapies (Huang *et al.* 2013, Larocca *et al.* 2013). In this regard, we and others have shown that the presence of high levels of brachyury in the primary tumor can predict poor prognosis in a range of human carcinomas including lung (Haro *et al.* 2013), hepatocellular (Du *et al.* 2014), GIST (Pinto *et al.* 2015), prostate (Pinto *et al.* 2014), colorectal (Kilic *et al.* 2011, Sarkar *et al.* 2012) and hormone receptor-positive breast cancer (Palena *et al.* 2014b). In agreement, here we have observed that high levels of brachyury in primary TNBC tumors associate with poor survival. However, it is important to point out that studies of the association between brachyury and patient survival were based on an arbitrary cutoff of ≥ 240 for the brachyury reactivity index. Thus, expression of brachyury in primary TNBC is proposed to be a bad prognostic indicator only for the fraction of tumors that express brachyury in at least 80% of the cancer cells and at high staining intensity (+++), which will result in reactivity index values ≥ 240 .

In our previous studies, we have conducted gain- and loss-of-function experiments to investigate the particular role of brachyury in breast carcinoma cells. Using basal-like, hormone-receptor-negative MDA-MB-436 cells, we have shown that silencing of brachyury is able to significantly reduce cell invasiveness and the tumor cells' ability to form mammospheres in primary and secondary cultures, a measure of tumor stemness, relative to their control counterparts. The loss of brachyury was also shown to significantly sensitize basal-like MDA-MB-436 cells to the cytotoxic activity of docetaxel, thus demonstrating a potential role for brachyury in tumor invasiveness and resistance to cell death in TNBC (Palena *et al.* 2014b). A recent report (Ben-Hamo *et al.* 2014) has identified brachyury as a dominant gene in a network of genes that most significantly discriminate TNBC from non-TNBC samples. Here, we have further demonstrated the prevalent expression of brachyury in a panel of TNBC human breast carcinoma cell lines and validated its role as a driver of tumor EMT with MDA-MB-436 TNBC cells, in which brachyury silencing markedly reduced the expression of mesenchymal vimentin and fibronectin, whereas increased the expression of epithelial ZO1.

In this study, the expression of brachyury mRNA was shown to be predominant among TNBC vs triple-positive samples in the TCGA database, whereas that was not the case with other EMT drivers, including TWIST1, SNAI2 or ZEB2 mRNA. These results seemed to contradict the expected enrichment of EMT markers in TNBC samples. Although the reason for these observations is unknown at this time, we have previously observed that expression of TWIST1, SNAI1 and SNAI2 mRNA is detectable in normal tissues at levels comparable with those observed in tumors (Roselli *et al.* 2012, Palena *et al.* 2014b). As the mRNA analysis includes not only tumor cells but also surrounding normal tissues, one possible explanation for the absence of difference of expression of the EMT markers between different tumor types could be the simultaneous detection in adjacent normal cells. Unlike TWIST1, SNAI1 and SNAI2, we have previously shown that expression of brachyury mRNA is undetectable in normal breast tissues (Palena *et al.* 2014b).

Among genes that were differentially expressed between TNBC and other tumors was IL8, a chemokine that was previously associated with tumor stemness and EMT in breast cancer (Ginestier *et al.* 2010, Fernando *et al.* 2011, Palena *et al.* 2012). We have previously demonstrated that IL8 is able to significantly upregulate brachyury expression in breast cancer cells at the transcriptional level and that upregulation of brachyury leads to increased secretion of IL8 and overexpression of IL8 receptors. It thus establishes an autocrine feed-forward loop that sustains the mesenchymal, resistant phenotype. We hypothesize that the presence of this autocrine regulatory loop in TNBC could drive the expression of brachyury and, potentially, the acquisition of mesenchymal and stem-like features by the tumor cells.

Although there have been some discrepancies regarding the detection of brachyury protein in various types of carcinomas, we believe that these inconsistencies are due to the utilization of various antibodies possessing a range of affinity and specificity for the target protein brachyury. We and others have shown that brachyury can be detected in the nucleus and/or the cytosol of carcinoma cells, whereas its expression in chordomas is predominantly seen in the nucleus of the tumor cells (Pinto *et al.* 2014, Hamilton *et al.* 2015, Miettinen *et al.* 2015). In agreement with those previous observations, here we demonstrated that brachyury protein can be detected either in the nucleus and/or the cytosol of TNBC cells, although their expression is somehow linked with a positive correlative trend observed between the nuclear

and cytoplasmic reactivity index ($r=0.236$, $P=0.04$). Although the biological relevance of nuclear vs cytosolic brachyury localization in tumor cells remains unknown, it was interesting to observe in this study that nuclear (but not cytosolic) expression of brachyury is associated with clinical stage of disease and was more predominant in metastatic vs primary tumor tissues. This potentially indicates a role for brachyury in tumor progression when localized in the nucleus, where its transcriptional activity is expected to take place. Future studies are warranted to evaluate, in a larger cohort of TNBC patients, the potential association between nuclear vs cytoplasmic brachyury expression and clinical outcome.

Altogether our observations demonstrate the potential of brachyury as a target for the treatment of early or metastatic TNBC and provide rationale for using a brachyury-targeting vaccine approach for treatment of this disease.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author's contribution statement

D H H and C P carried out studies at the mRNA level, performed cell line-based assays data analysis and wrote the manuscript. M R, P F, L C, F C, M T and F G carried out the immunohistochemical analyses, performed data analysis and wrote the manuscript. C P and F G designed and supervised the study. All authors read and approved the final manuscript. D H Hamilton, M Roselli, C Palena and F Guadagni contributed equally to this work.

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