Am J Transl Res 2018;10(3):936-947
www.ajtr.org /ISSN:1943-8141/AJTR0072880

*Original Article*

# The embryonic transcription factor Brachyury confers chordoma chemoresistance via upregulating CA9

Jiao Jian[1*], Nanzhe Zhong[1*], Dongjie Jiang[1*], Lei Li[2], Yan Lou[1], Wang Zhou[1], Su Chen[1], Jianru Xiao[1]

[1]*Department of Orthopedic Oncology, Changzheng Hospital, Second Military Medical University, Shanghai, China;* [2]*Institute of Biomedical Sciences and School of Life Sciences, East China Normal University, China.* [*]*Equal contributors and co-first authors.*

Received September 24, 2017; Accepted February 21, 2018; Epub March 15, 2018; Published March 30, 2018

**Abstract:** Being a rare malignant bone tumor on the axial skeleton, chordoma is locally invasive and has a high rate of recurrence. Despite extensive studies, the mechanisms of chordoma recurrence after surgical intervention, as well as resistance to radiation and chemotherapy, remain elusive. In this study, primary chordoma cell lines PCH1 and PCH2 were established and characterized by chordoma specific markers. We found that the embryonic transcription factor Brachyury inhibits Paclitaxel induced apoptosis in different cells, including PCH1 and U2OS cells. T gene regulated genes were identified in PCH1 and U2OS using microarray. After comparing gene regulated by Brachyury in different cells and the chromatin immunoprecipitation assay, we identified carbonic anhydrase IX (CA9) as a common target gene of Brachyury. Besides, immunohistochemical staining of CA9 and Brachyury in chordoma tissues revealed that their expression levels were positively correlated. We further showed that CA9 is responsible for Paclitaxel resistance in PCH1 cell. Our data suggest that CA9 plays a role in Brachyury mediated Paclitaxel resistance and serves as a potential target for chordoma treatment.

**Keywords:** Chordoma, Brachyury, carbonic anhydrase IX, chemoresistance

## Introduction

Chordoma is a rare bone tumor that preferentially affects bones of the skull base and spine. Chordomas arise from remnants of the embryonic notochord, the main embryonic axial structure that precedes the backbone, and have histological features and express characteristic genes of notochord cells [1]. Notochord cells normally persist after birth in the vertebral axis and skull base. Rarely, these cells undergo malignant transformation that leads to the formation of chordomas [2].

Chordomas are both sporadic and hereditary. The Brachyury protein, which has a critical role in notochord formation, is encoded by the T gene and has recently gained much attention due to its association with familial chordoma [3] and its role in epithelial-mesenchymal transition (EMT). The brachyury mutation was first described in mice by Nadine Dobrovolskaïa-Zavadskaïa as a mutation that affected tail length [4]. Mouse embryos lacking the T gene failed to form notochords, posterior regions, and the allantois; these embryos died at approximately 10 days of gestation. The mouse T gene was first cloned by Herrmann et al., encodes a 436-amino acid protein [5], and is highly conserved among vertebrates. The zebrafish *no tail* gene mutant phenocopied mouse brachyury mutant embryos as they lacked a differentiated notochord and caudal region [6].

Brachyury binds to a specific DNA sequence (TCACACCT) through its N terminal domain termed the T-box [7]. In addition to its function in early embryonic development, Brachyury can play a crucial role in tumorigenesis. Brachyury regulates the EMT in human lung tumors through repressing E-cadherin expression [8, 9]. Brachyury maintains the “plastic-state” of colorectal cancer stem cells by regulating NANOG expression [10]. Recently, much attention has been given to its function in chordomas. Brachyury is a specific marker for notochord-derived cancer and is a diagnostic marker for chordoma [11]. A common single-nucleotide polymorphism (SNP) in brachyury is strongly associated with chordoma [12]. The chromosomal region of 6q27 containing the T gene was duplicated in familial chordoma [3]. The chromosome re-

gion 7q33 is also linked with familial chordoma [13].

The treatment of chordomas is challenging with surgical removal of the tumor tissue being the first choice [14]. A recent report indicated efficacy of targeted therapy on chordomas [15]. However, the rate of local recurrence in chordoma patients remains high due to resistance to current chemotherapies. Some genes participate in chordoma chemotherapy resistance such as hypoxia-inducible factor 1 (*HIF1)* and multidrug resistance-associated protein 1 (*MRP1)* [16]. Brachyury is involved in chemotherapy resistance in some cancer stem cells. Knockdown of Brachyury increases the sensitivity of adenoid cystic carcinoma cells to chemotherapy and radiation [17].

Despite extensive research, the molecular mechanism underlying chordoma chemoresistance is still elusive. In our study, we used a chordoma cell line to investigate the function of the embryonic transcription factor Brachyury in regulating sensitivity to Paclitaxel. Downregulating Brachyury expression rendered the cell line more sensitive to Paclitaxel-induced apoptosis. Genes regulated by Brachyury were identified by microarray analysis. Furthermore, we provide evidence that carbonic anhydrase 9 (*CA9/CAIX*) serves as a Brachyury target gene and is involved in the resistance of chordomas to Paclitaxel-induced apoptosis.

## Materials and methods

### *Establishment of PCH1 (Patient-derived Changzheng Chordoma 1) cell lines and culture of tumor cell lines*

Chordoma samples were obtained intraoperatively from patients with no history of adjuvant therapies prior to surgery. Patients provided informed consent for the use of the tumor samples for investigational and analytical studies under the Changzheng Institutional Review Board guidelines. Samples were placed on ice immediately following surgical resection and transported to the laboratory. Tumor tissues were finely minced, extensively washed with PBS, transferred to 100-mm culture dishes, and cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco) with 10% fetal bovine serum (FBS) at 37°C with 5% $CO_2$. Cells were passed every five days for the first 3-6 passages.

U2OS, H293T and H460 cells were purchased from the American Type Culture Collection and maintained in DMEM and RPMI-1640 medium, respectively, supplemented with 10% FBS (Gibco) and 1% streptomycin/penicillin with 5% $CO_2$.

### *Cell counting Kit-8 (CCK-8) assay*

CCK-8 assay kits were purchased from Dojindo Molecular Technologies, Inc. and used according to the manufacturer's protocol.

### *mRNA isolation and semiquantitative reverse transcription (RT)-PCR*

To evaluate mRNA expression levels, total RNA was isolated using TRIzol reagent (Invitrogen). Reverse transcriptase (Promega) was employed for oligo(dT)-primed first-strand cDNA synthesis. For semiquantitative RT-PCR analysis, 2 μg of RT products were mixed with a volume of 25 μl containing 0.25 mM of each dNTP, 1.5 mM $MgCl_2$, 1.25 U Taq DNA polymerase (Thermo Fisher Scientific), 1× Taq reaction buffer (Promega), and 0.5 mM upstream and downstream PCR primers (**Table 1**). PCR products were visualized on agarose gels in 0.5× Tris-borate-EDTA buffer with ethidium bromide.

### *Virus infection*

Lentivirus constructs for RNA interference (RNAi) to reduce Brachyury levels and for T gene overexpression (TOE) (NM_003181.3) were purchased from Shanghai Research & Science Company. The RNAi sequence obtained from Sigma Aldrich for the short hairpin T gene (shT) was CCGGCGAGGAGATCACAGCTCTTAACTCGAGTTAAGAGCTGTGATCTCCTCGTTTTT. DNA was synthesized by Invitrogen. Short interfering RNAs (siRNAs) against *CA9* were siCA9#1: GATCTACCTACTGTTGAGGCT and siCA9#2: CAGCCGCTACTTCCAATATGA (Shanghai GenePharma). Virus infection was performed according to the manufacturer's protocol.

### *mRNA microarray assay*

Total RNA was quantified using the NanoDrop ND-2000 (Thermo Scientific). The RNA integrity was evaluated by Agilent Bioanalyzer 2100 (Agilent Technologies). The sample labeling, microarray hybridization and washing were performed based on the manufacturer's protocols. Briefly, total RNA was transcribed to double strand cDNA before being synthesized into cRNA and

**Table 1.** DNA primers used in the current study

| Gene | Forward primer | Reverse primer |
|---|---|---|
| Brachyury | TATGAGCCTCGAATCCACATAGT | CCTCGTTCTGATAAGCAGTCAC |
| CD24 | GCTCCTACCCACGCAGAT | GTGGCATTAGTTGGATTTGG |
| Col2a1 | GCTCCCAGAACATCACCTACC | TGAACCTGCTATTGCCCTCT |
| CA3 | AACCAGTCGCCCGTTGAG | TCGGCAGGTCTTCCCATT |
| KRT19 | GTGACATGCGAAGCCAATAT | TGAGCCGCTGGTACTCCT |
| HHIP | CTGCTTCTGTATTCAGGAGGTT | CGATAGCCCACCGTTCTT |
| NRARP | TCTTCCAGGAGGCTGTGCG | CAGGTTGCCGTCGATGACC |
| EPHA5 | AATGCCCTTCTGTGGTAC | CTTCATATCCTGCCTTGC |
| MEST | CAGGATGAGGGAGTGGTG | CTTGGTAGAAGATACGCAGT |
| LCP1 | GCACCCAACACTCCTATT | AATGTCAGCAAACAACCC |
| GATABP2 | ATGAAGATGGAAAGTGGCAGTC | TCGCTTGGGCTTGATGAGT |
| Bcl11a | GAGCACAAACGGAAACAATG | CTCTGGCGTGACCTGGAT |
| MERTK | TGAGGCAAGGGAAGAAGC | AAGGCAAGAGGCGGTAGGG |
| ELOVL2 | TTGGTGGTACTATTTCTCC | TTCTTCACTTCTTTCCCT |
| CA9 | GCTGCTTCTGGTGCCTGTC | GGAGCCCTCTTCTTCTGATTTA |
| TPD52 | AAGAGGAGCAGGAAGAGC | CTGAGCCAACAGACGAAA |
| WISP-2 | CTGTGGCTGCTGCCGGGTAT | CCAGGCCGTGCTCCATTCT |
| Bcl6coR | GCACTGCTCCCTCATCCT | CGACACTGACCCTGAAACG |
| CTCF | ACTGCGATAAGACCTTCC | CTTCGTTTCTCCTCCATT |
| v-myc | ACAGCGTCTGCTCCACCT | CCTCATCTTCTTGTTCCTCCT |
| Wnt3a | TCCACGCCATTGCCTCAG | CACCATCCCACCAAACTCG |
| RASdxmsI1 | AGCGAGATGAGCCCAGACC | GTGCCACGATGCCAAAGG |
| CXCL1 | CCCCAAGAACATCCAAAGTG | GATGCAGGATTGAGGCAAG |
| CXCL3 | CAGGAGCGTCCGTGGTCA | GGGATGCGGGGTTGAGAC |

labeled with Cyanine-3-CTP. The labeled cRNAs were then hybridized onto the microarray. After washing, the Agilent Scanner G2505C (Agilent Technologies) were used for the scanning of arrays. Feature Extraction software (version 10.7.1.1, Agilent Technologies) was applied to analyze array images to acquire raw data. Genespring were employed to finish the basic analysis of the raw data. The raw data was first normalized with the quantile algorithm. The probes that at least 100% of the values in any 1 out of all conditions have flags in "Detected" were selected for further analysis. Genes with differential expression were then identified by both fold change and *P* value calculated with t-test. A fold change ≥ 2.0 and a *P* value ≤ 0.05 was set as the threshold for up- and down-regulated genes.

*Chromatin immunoprecipitation (ChIP) assays*

H293T cells were transfected with pcDNA3.1-flag-Brachyury. To obtain DNA fragments of 100-1000 bp, chromatin of the pretreated H293T cells were cross-linked and sonicated. Cellular debris was discarded after centrifugation. Lysates were diluted in ChIP dilution buffer and incubated with salmon sperm DNA/protein A agarose slurry (Sigma) to remove nonspecific background. Chromatin solutions were sequentially incubated with RNA Pol II antibody (Santa Cruz Biotechnology) and anti-flag antibody (Sigma) and rotated overnight at 4°C. To pellet the beads, the solution was centrifuged (700 rpm). IgG was used as a negative control. The remaining proteins were digested by proteinase K (Invitrogen). DNA was extracted using phenol/chloroform/isoamyl alcohol and precipitated with 0.1 volume of 3 M sodium acetate and 2 volumes of ethanol with glycogen. Ten specific primers (**Table 1**) flanking potential binding sites within the *CA9* promoter were designed for PCR amplification of the genomic fragments. PCR products were separated using 2.0% agarose gel electrophoresis.

*Western blot analysis*

Cells were washed with PBS and cellular lysates were prepared using RIPA lysis buffer (Thermo Fisher Scientific). Protein samples were resolved on 10% SDS-denatured polyacrylamide gels. Immunoblotting was performed as previously described [18] and the following antibodies were used: rabbit anti-Brachyury antibody (Santa Cruz Biotechnology, cat. # sc-20109), rabbit anti-CA9 antibody (Santa Cruz Biotechnology, cat. # sc-25599), and mouse anti-β-actin antibody (Sigma Aldrich, cat. # A1978). Blots were visualized using an Odyssey scanner (Li-Cor).

*Immunohistochemical (IHC) staining*

Formalin-fixed, paraffin-embedded 3-μm tissue sections of 50 chordoma samples were cut. Two adjacent sections were stained with Brachyury or CA9 primary antibody, respectively. Immunostaining was performed using standard protocols. Samples were deparaffined deparaffinized in xylol and rehydrated in a graded ethanol series. Sections were immersed in 1 μM EDTA buffer (pH 8.0) and heat-induced epitope

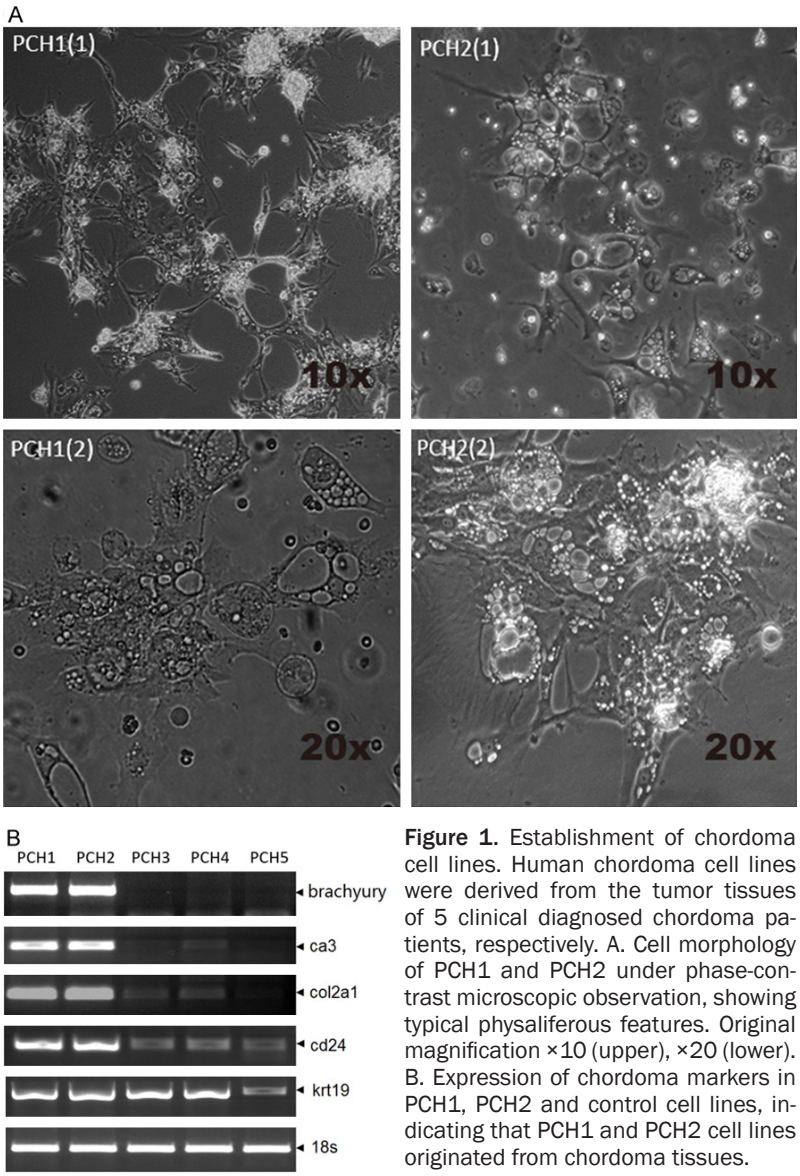


**Figure 1.** Establishment of chordoma cell lines. Human chordoma cell lines were derived from the tumor tissues of 5 clinical diagnosed chordoma patients, respectively. A. Cell morphology of PCH1 and PCH2 under phase-contrast microscopic observation, showing typical physaliferous features. Original magnification ×10 (upper), ×20 (lower). B. Expression of chordoma markers in PCH1, PCH2 and control cell lines, indicating that PCH1 and PCH2 cell lines originated from chordoma tissues.

retrieval was performed by microwaving samples for 25 minutes. Endogenous peroxidase activity was blocked by immersing samples in 0.3% methanolic hydrogen peroxide solution for 15 min. Slides were incubated in normal nonimmune serum for 30 min to reduce nonspecific conjugation of antibodies. Brachyury primary antibodies (1:500) and CA9 primary antibodies (1:100) were added to the adjacent slides, respectively and incubated overnight at 4°C. Slides were stained using an ABC kit (Sanjian Biotechnology, Inc) and then counterstained with hematoxylin. To ensure specificity of the IHC staining, sections in which the primary antibody was omitted served as the negative control.

To evaluate CA9 and Brachyury immunoreactivity, two independent pathologists chose representative visual fields under 200× magnification and counted the percentage of positively stained cells. The immunoactivity of CA9 was evaluated as follows: score 0, no stained cells; score 1+, focally positive staining or weakly positive in less than 25% of the cells; score 2+, moderately positive staining in more than 25% of the cells; and score 3+, strongly positive staining in more than 25% of the cells. For Brachyury, the intensity of positive staining was scored based on scores of 0 to 3 (0, no observable immunostaining; 1 mild brown staining; 2, moderate brown staining; and 3, dark brown staining). The percentage of positively stained cells was also scored based on the following criteria: 0, no staining; 1, less than 25% of the tumor cells were positively stained; 2, 25-75% of the tumor cells were positively stained; and 3, more than 75% of the tumor cells were positively stained. Five high-powered fields were selected by both pathologists to examine and calculate the immunoreactivity of cells. The scores of immunoactivity and staining positivity were then added to give the expression score of the sample. The final expression score was defined as following: null (-, total score = 0), weak (+, total score = 1-3), moderate (++, total score = 4-5), and strong (+++, total score ≥ 6).

*Flow cytometric analysis*

Apoptosis assays were performed using annexin V-fluorescein isothiocyanate (annexin V-FITC) and propidium iodide (PI) (BD Pharmingen) according to the manufacturer's protocol. Briefly, PCH1 and H460 cells were centrifuged, washed

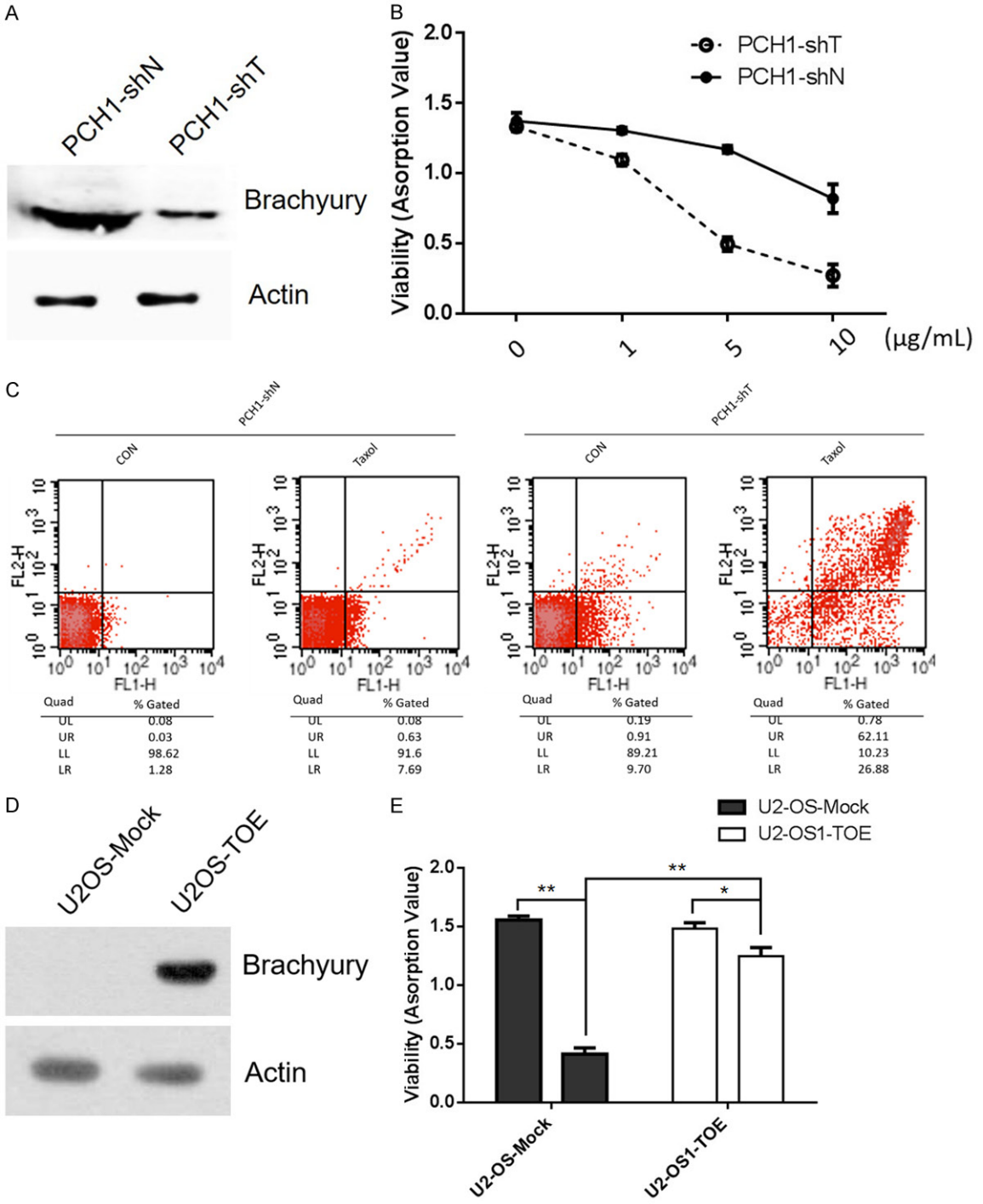


**Figure 2.** Brachyury protected cell from Paclitaxel induced apoptosis. A. T gene was down-regulated by lenti-viral transfected shRNA (PCH1-shT). β-actin was used as loading control. B. PCH1-shT and the control cell were treated with different concentrations of Paclitaxel. Cell viability was measured using CCK-8 assay, showing PCH1-shT was more vulnerable to Paclitaxel than the control cell. C. PCH1-shT and the control cell were treated with paclitaxel (5 μg/mL). Cells were fixed and stained with AnnexinV and Propidium Iodide (PI) after 24 hours' Paclitaxel treatment. Percentage of apoptotic cell was analyzed by FACS. PCH1-shT had higher level of apoptosis than the control cell. D. T gene was overexpressed in U2OS cell. β-actin was used as loading control. E. Brachyury protects U2OS from Paclitaxel induced apoptosis. U2OS-mock and U2OS-TOE cells were treated with Paclitaxel for 48 hours. Cell viability was measured with CCK-8 assay. The values were presented as mean ± SD. **P<0.01; *P<0.05. All experiment was repeated for at least three times.

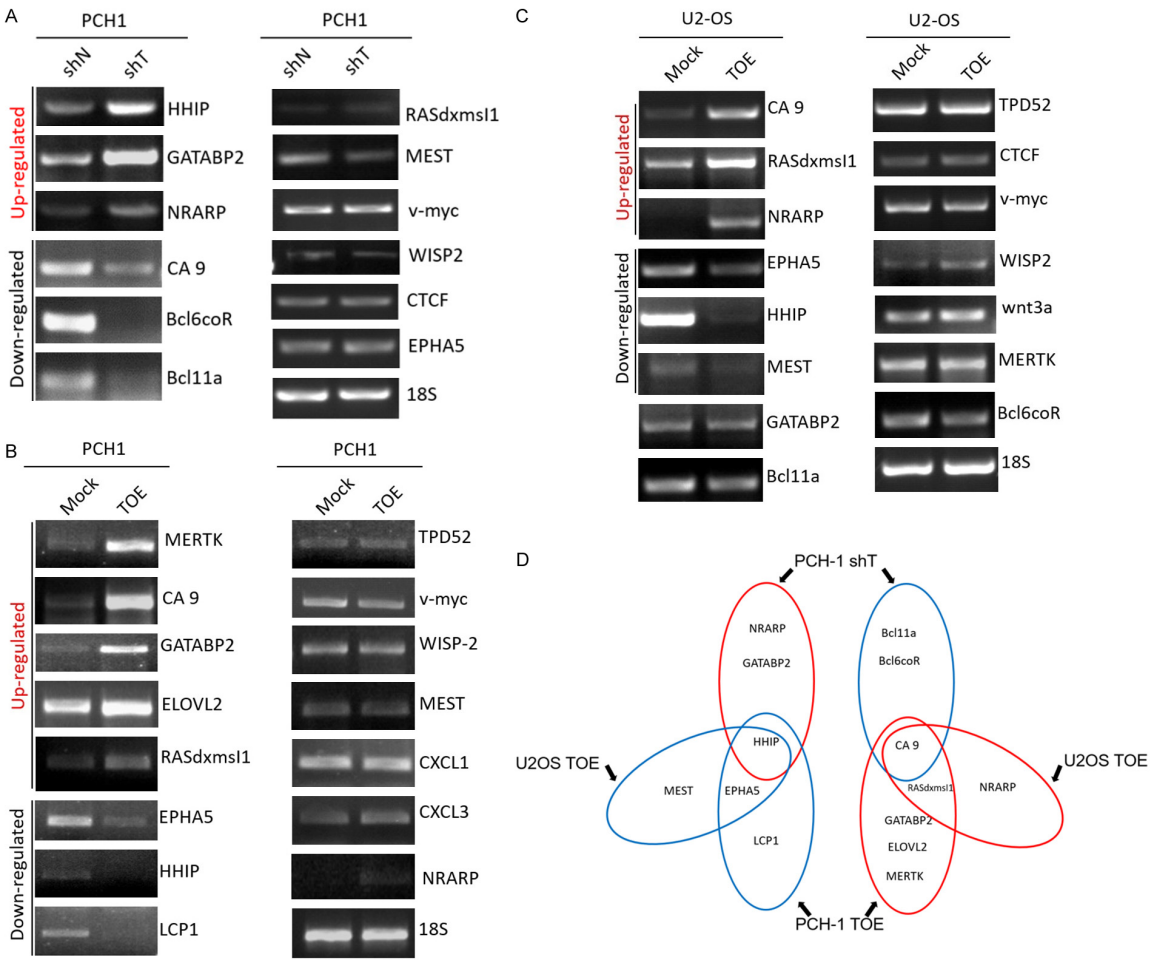


**Figure 3.** Brachyury-targeting genes in PCH1 cells. A. Validation of expression changes of representative genes detected in microarray analysis by RT-PCR in PCH1 negative control cells (shN) and Brachyury knockdown PCH1 cells (shT). B. Validation of expression changes of representative genes by RT-PCR in brachyury overexpression PCH1 cell. C. Validation of expression changes of representative genes by RT-PCR in U2OS cell with Brachyury overexpression. D. A graphic summary of the alteration of gene expression shows that CA9 and HHIP might be the best possibility to serve as the target regulated by T gene. The genes in the blue ellipse were down-regulated, while the genes in the red ellipse were up-regulated.

with cold binding buffer, and resuspended in 200 μl binding buffer. FITC-conjugated annexin V (10 μl) was added to each sample following incubation at 4°C in the dark for 15 min. Cells were washed and PI (10 μl) was added to the mixture following a 5-min incubation. Cells were immediately subjected to flow cytometric analysis on a FACS Calibur (BD Biosciences). The percentage of early and/or late apoptotic cells in each group was determined.

*Statistics*

SPSS 18.0 analysis software was applied for all statistical analyses and graphs. All data from cell-based experiments were obtained from at least three independent experiments and were recorded as the mean ± standard deviation (SD). To compare means of more than two groups, one-way ANOVA was used; for other results, differences between groups were evaluated using the Student's t test. The association between Brachyury immunoreactivity and CA9 immunoreactivity was evaluated using Spearman's rank tests. *P* values < 0.05 were considered statistically significant.

## Results

*Generation of chordoma cell lines*

To investigate the molecular mechanism underlying chordoma chemotherapy resistance, we generated two primary cell lines from human

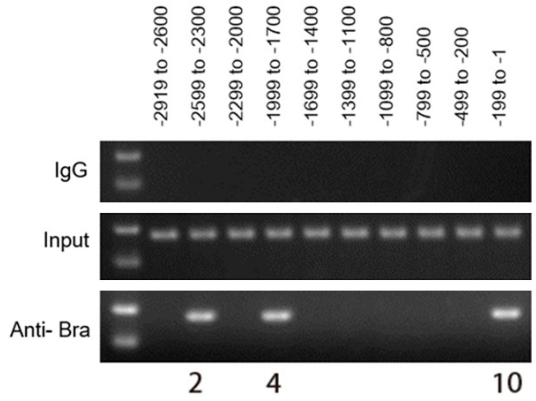


**Figure 4.** Transcriptional regulation of CA9 by T gene. ChIP assay was carried out using nuclear extracts from the H293T cells with the transfection of pcDNA3.1-Flag-Brachyury plasmid and an antibody against Flag, followed by RT-PCR analysis. Primers were designed for the 10 parts of the 5' CA9 promoter sequence (-1/-2919 bp). IgG was taken as a negative control. DNA fragments corresponding to the 2, 4 and 10 segment of promoter sequence (the -2699 bp to -1700 bp and the -199 bp to -1 bp region) were specifically pulled down.

tumor tissues. Two cell lines, PCH1 and PCH2, were successfully generated (**Figure 1A**). RT-PCR results indicated that these two cell lines expressed the chordoma-specific markers brachyury, ca3, col2a1, cd24, and krt19 [19] (**Figure 1B**). These markers were detected at only low levels in three other primary cell lines PCH3, PCH4, and PCH5. In addition, we examined T gene expression in other cancer cell lines and found it was expressed in H460 cells, but not in U2OS cells (Figure S1). Thus, two chordoma cell lines were successfully generated to assess the molecular mechanism underlying the biological behavior of chordomas.

*T gene knockdown confers Paclitaxel sensitivity*

Previous studies suggest that knockdown of Brachyury increases the sensitivity of adenoid cystic carcinoma cells to chemotherapy and radiation [20]. A recent clinical study indicated that Brachyury is expressed in more than 90% of chordomas [21]. Therefore, we investigated whether Brachyury also plays a prominent role in the resistance of chordomas to common chemotherapies such as Paclitaxel.

We examined cell viability following reduced expression or forced overexpression of T gene. Brachyury protein levels were efficiently reduced by siRNA in PCH1 cells (**Figure 2A**). When PCH1 cells were treated with different concentrations of Paclitaxel, cell viability of control PCH1 cells and Brachyury-knockdown cells were decreased in a dose-dependent manner. Brachyury knockdown sensitized the cells to Paclitaxel treatment, indicating that Brachyury may protect the cells from Paclitaxel-induced apoptosis (**Figure 2B**). This result was further validated by flow cytometric analysis. Flow cytometric data indicated that 62% of Brachyury-knockdown cells went through apoptosis, whereas only 0.63% of control PCH1 cells went through apoptosis (**Figure 2C**). Similar results were obtained in H460 cells (Figure S2A and S2B).

Because U2OS cells lack Brachyury expression (Figure S1), we generated a U2OS cell line with forced overexpression of T gene (**Figure 2D**). Brachyury overexpression made U2OS cells more resistant to Paclitaxel treatment (**Figure 2E**). Taken together, knockdown of Brachyury sensitized cells to Paclitaxel treatment and overexpression of Brachyury induced resistance.

*Brachyury regulated genes in the PCH1 cell line*

We hypothesized that Brachyury target genes are involved in Paclitaxel-induced apoptosis because Brachyury is a transcription factor. Thus, we examined global changes in gene expression between the control cell line and a Brachyury-knockdown cell line using microarray analysis (Shanghai OE Biotech Co. Ltd.). A total of 1472 genes were significantly altered by at least 2-fold. Among these genes, 625 genes were upregulated and 847 genes were downregulated in the Brachyury-knockdown H460 and PCH1 cell lines (GEO accession numbers: GSE103716, GSM2779628, GSM2779629, GSM2779630, GSM2779631, GSM2779632, GSM2779633, GSM2779634). The genes with substantially altered expression and relating to tumorigenesis were further confirmed by RT-PCR. After the inhibition of Brachyury expression in PCH1 cells, expression changes in these genes were detected, including *HHIP, GATABP2, NRARP, CA9, Bcl6cor* and *Bcl11a* (**Figure 3A**). We also examined gene expression in PCH1-TOE and U2OS-TOE cells. Increased expression of *CA9, ELOVL2, RASdxmsl1, GATABP2*, and *MERTK* was seen in the PCH1-TOE cell line and expression of *CA9, NRARP,* and *RASdxmsl1* was increased in the U2OS-TOE cell line (**Figure**

**Table 2.** The immunostaining score of CA9 and Brachyury expression

| | CA9 expression score | | | | Total | *P* | *r* |
|---|---|---|---|---|---|---|---|
| | 0 (n=7) | 1 (n=14) | 2 (n=14) | 3 (n=15) | | | |
| Brachyury expression score | | | | | | <0.001 | 0.634 |
| - | 4 | 4 | 0 | 0 | 8 | | |
| ++ | 2 | 6 | 3 | 1 | 12 | | |
| +++ | 1 | 2 | 8 | 8 | 19 | | |
| ++++ | 0 | 2 | 3 | 6 | 11 | | |

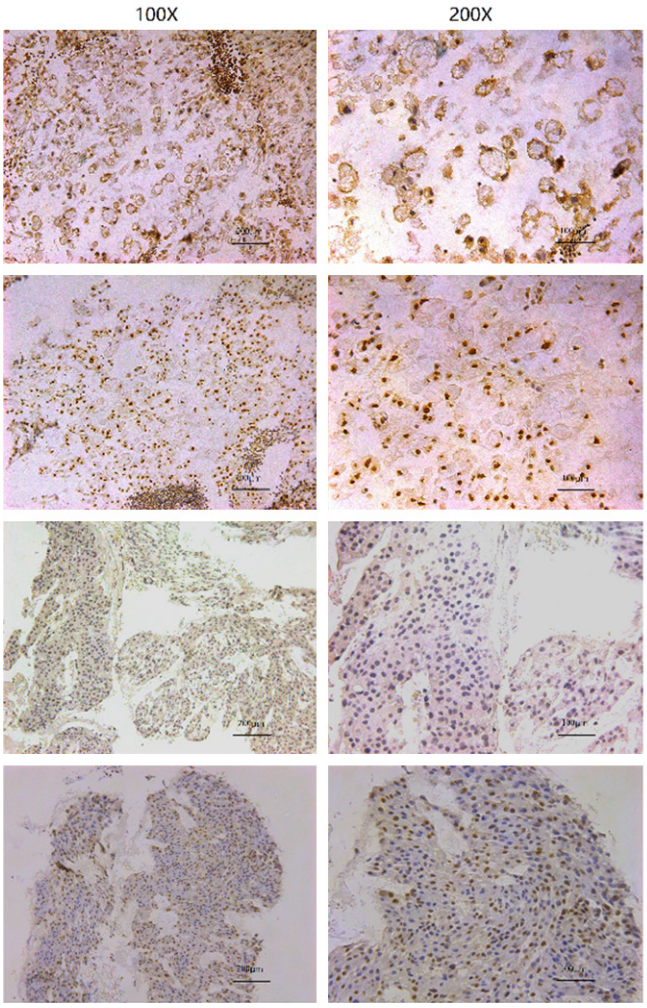


**Figure 5.** IHC staining of Brachyury and CA9. IHC staining was performed on the adjacent sections of the same sample to evaluate the correlation between Brachyury and CA9. The strong staining of Brachyury in one chordoma case which also exhibited strong staining of CA9 (the upper panel); reduced staining of Brachyury in one chordoma case, which also displayed weak staining of CA9 (the lower panel). In addition, non-parametric Spearman correlation tests were used to statistically analyze the correlation of Brachyury and CA9 expression score, with results showed in **Table 2**.

**3B** and **3C**). Taken together, our results indicate that *CA9* and *HHIP* are the most likely regulatory targets of brachyury (**Figure 3D**).

*CA9, a Brachyury target gene, mediated Paclitaxel sensitivity in PCH1 cells*

Brachyury expression altered the sensitivity of both PCH1 and U2OS cells to Paclitaxel, indicating that this activity was not cell-type dependent. Subsequently, efforts were made to explore the target genes of Brachyury involved in Paclitaxel sensitivity. Therefore, we compared the gene expression profiles of different cells following Brachyury overexpression or knockdown.

The Brachyury-induced altered expression was consistent for some genes between the different cell lines. For example, *HHIP* and *EPHA5* expression was decreased and *CA9* and *Radsxmsl* expression was increased following forced

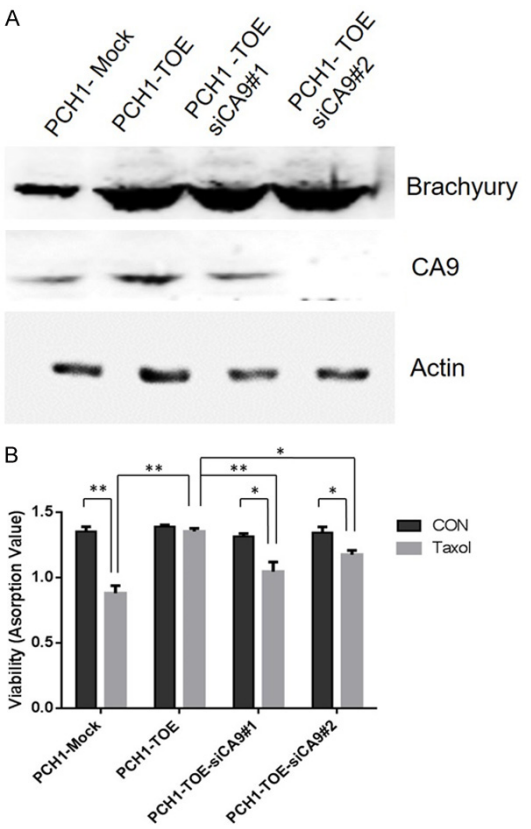


**Figure 6.** CA9 protects PCH1 from paclitaxel induced apoptosis. A. Western blot analysis of Brachyury, CA9 and β-actin in the transfected PCH1 cells, which were used for the cell viability assay. B. CA9 protects PCH1 from Paclitaxel induced apoptosis. Cell viability was measure by CCK-8 assay. Each experiment was repeated three times. *indicates *P*-value <0.05 and **indicates *P*-value <0.01.

Brachyury expression in PCH1 and U2OS cells. GO analysis and previous studies suggested that *CA9* is an apoptosis-related gene [22, 23]. Aomatsu et al. demonstrated that CA9 levels can predict the response of breast cancer patients to chemotherapy [24]. Moreover, inhibition of CA9 facilitated agent-mediated apoptosis in human cancer cells [25].

To explore whether Brachyury regulates *CA9* transcription, a ChIP assay was performed with the *CA9* promoter in H293T cells transfected with the pcDNA3.1-Flag-Brachyury vector. The 5' *CA9* promoter sequence (-2919 bp to -1 bp) was divided into 10 segments for the designation of 10 primer pairs to amplify each segment of the promoter. DNA fragments corresponding to the -2699 bp to -1700 bp and -199 bp to -1 bp regions were specifically pulled down by the anti-Flag Tag antibody. This was not seen when an irrelevant IgG was used as a control (**Figure 4**). IHC analyses also indicated a positive correlation between Brachyury and CA9 expression ($P < 0.001$, $r = 0.634$) (**Table 2**; **Figure 5**).

To further uncover whether CA9 is involved in Brachyury-mediated Paclitaxel sensitivity, *CA9* expression was downregulated using RNAi in Brachyury overexpressing cells (**Figure 6A**). CA9-knockdown abolished the protective effect of Brachyury on Paclitaxel treatment (**Figure 6B**). Thus, CA9 takes part in Brachyury mediated Paclitaxel resistance.

## Discussion

Chordoma is a rare bone cancer. After birth, some notochord cells remain in bones at the base of the skull, in vertebrae, and in the tail bone. Rarely, these cells undergo changes that give rise to a chordoma [26]. Surgical resection remains the primary treatment for chordomas as chemotherapy resistance is common [1]. Identification of factors or signal pathways involved in the resistance of chordomas to chemotherapy is necessary to improve patient prognoses. Therefore, we generated primary chordoma cell lines that express chordoma markers. As seen previously [27], high levels of Brachyury expression was observed in these cell lines. However, primary cell lines have limits as they are usually overwhelmed by fibroblasts after 3-6 passages. Therefore, the primary chordoma cell lines were only used for experiments at passages two to six.

Being a transcription factor, Brachyury plays a critical role in cancer development. Brachyury induces EMT, a critical step in carcinoma metastasis, by repressing E-cadherin expression [9]. The role of Brachyury in cancer chemo-resistance has been studied recently. Brachyury knockdown not only inhibits migration and invasion by adenoid cystic carcinoma stem cells, but also suppresses chemo-resistance by downregulating drug transporter genes such as the ATP-binding cassette transporters [17]. Brachyury can bind to a half T-box consensus site in the promoter region of the p21 gene and influence the sensitivity of carcinomas to chemotherapy [28].

Although Brachyury expression levels are closely associated with chordoma viability [29], its effects on chordoma chemoresistance has not

yet been described. Here, we investigated its function in chemoresistance using primary chordoma cell lines. The chordoma cell lines became sensitive to Paclitaxel-induced apoptosis when Brachyury expression was knocked down. Brachyury overexpression conferred a survival advantage for chordoma cells upon treatment with Paclitaxel. This observation was not confined to chordoma cells because U2OS cells exhibited decreased sensitivity following overexpression of Brachyury levels. These results were consistent with the previous study which showed a similar role of Brachyury in adenoid cystic carcinoma cells [20]. These data indicate that Brachyury functions in chordoma chemoresistance. Overexpressed in most chordomas [30], Brachyury may be a critical molecular mechanism underlying the insensitivity of chordomas to current chemotherapies. As a transcription factor with broad effects on both mesoderm formation and carcinogenesis, Brachyury may participate in this process by regulating the expression of specific genes.

In our study, some common genes targeted by Brachyury, including *CA9*, were identified in both PCH1 and U2OS cells. Carbonic anhydrases are a large family of zinc metalloenzymes that catalyze the reversible hydration of carbon dioxide [31]. Under normal conditions, CA9 can preserve the acid-base balance via its enzymatic activity. However, under hypoxic conditions, CA9 expression is upregulated by hypoxia-induced factor 1 and enhances the acidification of the tumor environment, thus facilitating the survival of cancer cells [32]. Recent data revealed that zinc finger E-box-binding homeobox 1 can directly bind to the *CA9* promoter, transcriptionally regulating CA9 expression [33]. Here, ChIP analyses indicated that *CA9* transcription was directly regulated by Brachyury. IHC staining further supported the positive association between Brachyury and CA9. Therefore, Brachyury was identified as a new factor controlling CA9 expression.

Facilitating chemoresistance is considered a vital function of CA9 in tumorigenesis. High CA9 levels reduce the chemosensitivity of various malignancies [33-35] by disturbing the tumor microenvironment. CA9 inhibitors reduce growth and metastasis of several tumors [36, 37]. Similarly, our GO analysis indicated that CA9 is a key factor contributing to apoptosis, further suggesting that CA9 may be involved in Brachyury-mediated Paclitaxel resistance. Indeed, knockdown of CA9 in PCH1 cells reduced Paclitaxel resistance induced by Brachyury. Thus, CA9 may also be a drug target for chordoma therapy.

This study provides evidence that *CA9* is a Brachyury target gene. Furthermore, CA9 is implicated in Brachyury-mediated Paclitaxel sensitivity, implying that CA9 participates in chemotherapy resistance of cancers, including chordoma. Further studies are required to define the mechanisms underlying CA9 involvement in chordoma chemoresistance and its value in chordoma therapy.

## Acknowledgements

We are grateful to the patients for donating their tissues for research and to all of the clinicians and support staff who were involved in caring for the patients involved in this study in the Department of Orthopedic Oncology, Shanghai Changzheng Hospital. The research was generously supported by the National Natural Science Foundation of China (Grant No. 8110-2036), the Shanghai Sailing Program (18YF-1423000), the Foundation for Young Scholars of Second military Medical University (2017QN-16, 2917QN17).

## Disclosure of conflict of interest

None.

**Address correspondence to:** Su Chen and Jianru Xiao, Department of Orthopedic Oncology, Changzheng Hospital, Second Military Medical University, 415 Fengyang Road, Shanghai 200003, China. Fax: +86 2163720099; E-mail: chensudx@126.com (SC); jianruxiao83@163.com (JRX)

## References

[1] Walcott BP, Nahed BV, Mohyeldin A, Coumans JV, Kahle KT and Ferreira MJ. Chordoma: current concepts, management, and future directions. Lancet Oncol 2012; 13: e69-e76.

[2] Nibu Y, Jose-Edwards DS and Di Gregorio A. From notochord formation to hereditary chordoma: the many roles of Brachyury. Biomed Res Int 2013; 2013: 826435.

[3] Yang XR, Ng D, Alcorta DA, Liebsch NJ, Sheridan E, Li S, Goldstein AM, Parry DM and Kelley MJ. T (brachyury) gene duplication confers major susceptibility to familial chordoma. Nat Genet 2009; 41: 1176-1178.

[4] Korzh V and Grunwald D. Nadine Dobrovolskaia-Zavadskaia and the dawn of developmental genetics. Bioessays 2001; 23: 365-371.
[5] Herrmann BG, Labeit S, Poustka A, King TR and Lehrach H. Cloning of the T gene required in mesoderm formation in the mouse. Nature 1990; 343: 617-622.
[6] Schulte-Merker S, van Eeden FJ, Halpern ME, Kimmel CB and Nusslein-Volhard C. no tail (ntl) is the zebrafish homologue of the mouse T (Brachyury) gene. Development 1994; 120: 1009-1015.
[7] Muller CW and Herrmann BG. Crystallographic structure of the T domain-DNA complex of the Brachyury transcription factor. Nature 1997; 389: 884-888.
[8] Xu K, Liu B and Liu Y. Impact of Brachyury on epithelial-mesenchymal transitions and chemosensitivity in non-small cell lung cancer. Mol Med Rep 2015; 12: 995-1001.
[9] Sun S, Sun W, Xia L, Liu L, Du R, He L, Li R, Wang H and Huang C. The T-box transcription factor Brachyury promotes renal interstitial fibrosis by repressing E-cadherin expression. Cell Commun Signal 2014; 12: 76.
[10] Sarkar D, Shields B, Davies ML, Muller J and Wakeman JA. BRACHYURY confers cancer stem cell characteristics on colorectal cancer cells. Int J Cancer 2012; 130: 328-337.
[11] Vujovic S, Henderson S, Presneau N, Odell E, Jacques TS, Tirabosco R, Boshoff C and Flanagan AM. Brachyury, a crucial regulator of notochordal development, is a novel biomarker for chordomas. J Pathol 2006; 209: 157-165.
[12] Pillay N, Plagnol V, Tarpey PS, Lobo SB, Presneau N, Szuhai K, Halai D, Berisha F, Cannon SR, Mead S, Kasperaviciute D, Palmen J, Talmud PJ, Kindblom LG, Amary MF, Tirabosco R and Flanagan AM. A common single-nucleotide variant in T is strongly associated with chordoma. Nat Genet 2012; 44: 1185-1187.
[13] Kelley MJ, Korczak JF, Sheridan E, Yang X, Goldstein AM and Parry DM. Familial chordoma, a tumor of notochordal remnants, is linked to chromosome 7q33. Am J Hum Genet 2001; 69: 454-460.
[14] Williams BJ, Raper DM, Godbout E, Bourne TD, Prevedello DM, Kassam AB and Park DM. Diagnosis and treatment of chordoma. J Natl Compr Canc Netw 2013; 11: 726-731.
[15] Lebellec L, Aubert S, Zairi F, Ryckewaert T, Chauffert B and Penel N. Molecular targeted therapies in advanced or metastatic chordoma patients: facts and hypotheses. Crit Rev Oncol Hematol 2015; 95: 125-131.
[16] Ji Z, Long H, Hu Y, Qiu X, Chen X, Li Z, Fan D, Ma B and Fan Q. Expression of MDR1, HIF-1alpha and MRP1 in sacral chordoma and chordoma cell line CM-319. J Exp Clin Cancer Res 2010; 29: 158.
[17] Kobayashi Y, Sugiura T, Imajyo I, Shimoda M, Ishii K, Akimoto N, Yoshihama N and Mori Y. Knockdown of the T-box transcription factor Brachyury increases sensitivity of adenoid cystic carcinoma cells to chemotherapy and radiation in vitro: implications for a new therapeutic principle. Int J Oncol 2014; 44: 1107-1117.
[18] Wan Z, Jiang D, Chen S, Jiao J, Ji L, Shah AS, Wei H, Yang X, Li X, Wang Y and Xiao J. T-box transcription factor brachyury promotes tumor cell invasion and metastasis in non-small cell lung cancer via upregulation of matrix metalloproteinase 12. Oncol Rep 2016; 36: 306-314.
[19] Scheil-Bertram S, Kappler R, von Baer A, Hartwig E, Sarkar M, Serra M, Bruderlein S, Westhoff B, Melzner I, Bassaly B, Herms J, Hugo HH, Schulte M and Moller P. Molecular profiling of chordoma. Int J Oncol 2014; 44: 1041-1055.
[20] Kobayashi Y, Sugiura T, Imajyo I, Shimoda M, Ishii K, Akimoto N, Yoshihama N and Mori Y. Knockdown of the T-box transcription factor Brachyury increases sensitivity of adenoid cystic carcinoma cells to chemotherapy and radiation in vitro: implications for a new therapeutic principle. Int J Oncol 2014; 44: 1107-1117.
[21] Wang K, Tian K, Wang L, Wu Z, Ren C, Hao S, Feng J, Li J, Wan H, Jia G, Zhang L and Zhang J. Brachyury: a sensitive marker, but not a prognostic factor, for skull base chordomas. Mol Med Rep 2015; 12: 4298-4304.
[22] Dizaji MZ, Malehmir M, Ghavamzadeh A, Alimoghaddam K and Ghaffari SH. Synergistic effects of arsenic trioxide and silibinin on apoptosis and invasion in human glioblastoma U87MG cell line. Neurochem Res 2012; 37: 370-380.
[23] Yu SJ, Yoon JH, Lee JH, Myung SJ, Jang ES, Kwak MS, Cho EJ, Jang JJ, Kim YJ and Lee HS. Inhibition of hypoxia-inducible carbonic anhydrase-IX enhances hexokinase II inhibitor-induced hepatocellular carcinoma cell apoptosis. Acta Pharmacol Sin 2011; 32: 912-920.
[24] Aomatsu N, Yashiro M, Kashiwagi S, Kawajiri H, Takashima T, Ohsawa M, Wakasa K and Hirakawa K. Carbonic anhydrase 9 is associated with chemosensitivity and prognosis in breast cancer patients treated with taxane and anthracycline. BMC Cancer 2014; 14: 400.
[25] Cianchi F, Vinci MC, Supuran CT, Peruzzi B, De Giuli P, Fasolis G, Perigli G, Pastorekova S, Papucci L, Pini A, Masini E and Puccetti L. Selective inhibition of carbonic anhydrase IX decreases cell proliferation and induces ceramide-mediated apoptosis in human cancer cells. J Pharmacol Exp Ther 2010; 334: 710-719.
[26] Yakkioui Y, van Overbeeke JJ, Santegoeds R, van Engeland M and Temel Y. Chordoma: the

entity. Biochim Biophys Acta 2014; 1846: 655-669.
[27] Karikari IO, Gilchrist CL, Jing L, Alcorta DA, Chen J, Richardson WJ, Gabr MA, Bell RD, Kelley MJ, Bagley CA and Setton LA. Molecular characterization of chordoma xenografts generated from a novel primary chordoma cell source and two chordoma cell lines. J Neurosurg Spine 2014; 21: 386-393.
[28] Huang B, Cohen JR, Fernando RI, Hamilton DH, Litzinger MT, Hodge JW and Palena C. The embryonic transcription factor Brachyury blocks cell cycle progression and mediates tumor resistance to conventional antitumor therapies. Cell Death Dis 2013; 4: e682.
[29] Hsu W, Mohyeldin A, Shah SR, Ap RC, Johnson LF, Sedora-Roman NI, Kosztowski TA, Awad OA, McCarthy EF, Loeb DM, Wolinsky JP, Gokaslan ZL and Quinones-Hinojosa A. Generation of chordoma cell line JHC7 and the identification of Brachyury as a novel molecular target. J Neurosurg 2011; 115: 760-769.
[30] Miettinen M, Wang Z, Lasota J, Heery C, Schlom J and Palena C. Nuclear brachyury expression is consistent in chordoma, common in germ cell tumors and small cell carcinomas, and rare in other carcinomas and sarcomas: an immunohistochemical study of 5229 cases. Am J Surg Pathol 2015; 39: 1305-1312.
[31] Chadwick AL, Howell A, Sotgia F and Lisanti MP. Carbonic anhydrase 9 (CA9) and redox signaling in cancer-associated fibroblasts: therapeutic implications. Cell Cycle 2013; 12: 2534-2535.
[32] Koukourakis MI, Giatromanolaki A, Danielidis V and Sivridis E. Hypoxia inducible factor (HIf1alpha and HIF2alpha) and carbonic anhydrase 9 (CA9) expression and response of head-neck cancer to hypofractionated and accelerated radiotherapy. Int J Radiat Biol 2008; 84: 47-52.
[33] Zheng G, Peng C, Jia X, Gu Y, Zhang Z, Deng Y, Wang C, Li N, Yin J, Liu X, Lu M, Tang H and He Z. ZEB1 transcriptionally regulated carbonic anhydrase 9 mediates the chemoresistance of tongue cancer via maintaining intracellular pH. Mol Cancer 2015; 14: 84.
[34] O'Toole D, Couvelard A, Rebours V, Zappa M, Hentic O, Hammel P, Levy P, Bedossa P, Raymond E and Ruszniewski P. Molecular markers associated with response to chemotherapy in gastro-entero-pancreatic neuroendocrine tumors. Endocr Relat Cancer 2010; 17: 847-856.
[35] Williams E, Martin S, Moss R, Durrant L and Deen S. Co-expression of VEGF and CA9 in ovarian high-grade serous carcinoma and relationship to survival. Virchows Arch 2012; 461: 33-39.
[36] Lou Y, McDonald PC, Oloumi A, Chia S, Ostlund C, Ahmadi A, Kyle A, Auf DKU, Leung S, Huntsman D, Clarke B, Sutherland BW, Waterhouse D, Bally M, Roskelley C, Overall CM, Minchinton A, Pacchiano F, Carta F, Scozzafava A, Touisni N, Winum JY, Supuran CT and Dedhar S. Targeting tumor hypoxia: suppression of breast tumor growth and metastasis by novel carbonic anhydrase IX inhibitors. Cancer Res 2011; 71: 3364-3376.
[37] Said HM, Hagemann C, Carta F, Katzer A, Polat B, Staab A, Scozzafava A, Anacker J, Vince GH, Flentje M and Supuran CT. Hypoxia induced CA9 inhibitory targeting by two different sulfonamide derivatives including acetazolamide in human glioblastoma. Bioorg Med Chem 2013; 21: 3949-3957.

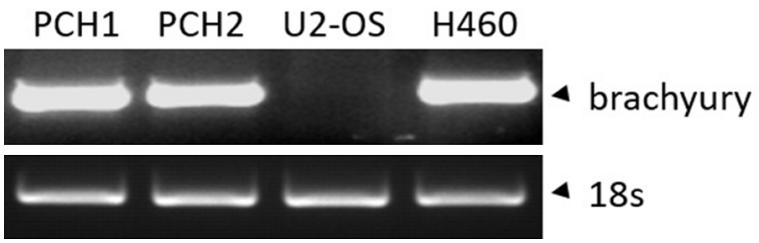


**Figure S1.** Brachyury expression in PCH1, PCH2, U2OS, H460 cell. mRNA was extracted from indicated cells. RT-PCR was performed to measure brachyury expression. 18S mRNA was an internal control.

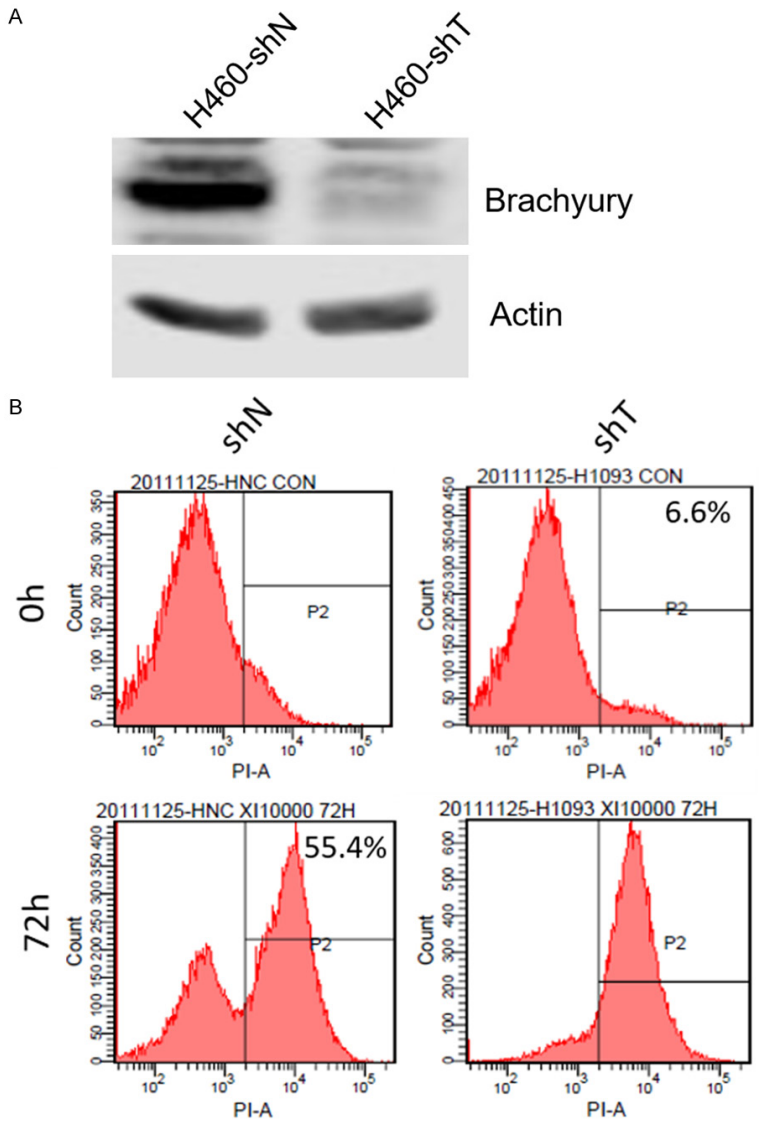


**Figure S2.** Brachyury overexpression protects H460 from paclitaxel induced apoptosis. A. Knockdown of Brachyury in H460 cell was validated by western blotting. β-actin was an internal control. B. Both the H460 control and knock-down cells were treated with 5 μg/mL paclitaxel for 72 hours. Cells were fixed and stained with AnnexinV only. Cell apoptosis was analyzed by FACS.