Original Article
The embryonic transcription factor Brachyury confers chordoma chemoresistance via upregulating CA9

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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-
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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. Downregulation of 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% CO₂. 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% CO₂.

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 MgCl₂, 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 CCGGCGAGGAGATCACAGCTCTTACTCGAGTTAAGAGCTGTGATCTCCTCGTTTTT. DNA was synthesized by Invitrogen. Short interfering RNAs (siRNAs) against CA9 were siCA9#1: GATCTACCTACTGTGAGCTCAGC and siCA9#2: CAGCCGCTACTTCAATATGA (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
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. Gene-spring 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. Lyastes were diluted in ChIP dilution buffer and incubated with salmon sperm DNA/protein A agarose slurry (Sigma) to remove non specific 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 protease K (Invtrogen). 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.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brachyury</td>
<td>TATGAGCCTCGAATCCACATGATG</td>
<td>CTCCTTCTGATAAGCACTGCACTCAGC</td>
</tr>
<tr>
<td>CD24</td>
<td>GCTCCCAACCCCGCAGATG</td>
<td>GTGGCCATTGATTGAGTTGGG</td>
</tr>
<tr>
<td>Col2a1</td>
<td>GCTCCCAAGAACATACCCATACCC</td>
<td>TGAACCTGATTTGCTGACCTCCTCCT</td>
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<td>CA3</td>
<td>ACCACTGTCGGCCTGGTGA</td>
<td>TCGGCAAGTCTGTCCTGCCATT</td>
</tr>
<tr>
<td>KRT19</td>
<td>GTAAGAGCAGGAGACATATG</td>
<td>TGAGCCCGTGTTACTCCT</td>
</tr>
<tr>
<td>HHIP</td>
<td>CTGGCTCTGTATGCAGATGTTGGTTG</td>
<td>CGATAGCCACCTGCCCTTT</td>
</tr>
<tr>
<td>NRARP</td>
<td>TCCTCCAGAGGGCTGGTGGA</td>
<td>CAGAGTTCGGCTGACCTGATGG</td>
</tr>
<tr>
<td>EPHA5</td>
<td>AATGCCCTCTCAGCTGTAC</td>
<td>CTTCATACCTCCTGCCCTC</td>
</tr>
<tr>
<td>MEST</td>
<td>CAGAGAGGGAGATTGTTG</td>
<td>CTGATGAGATAGACCCACTG</td>
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<tr>
<td>LCP1</td>
<td>GCACAAAGAATGAGACCTG</td>
<td>ATAGTCAGCAAAACACC</td>
</tr>
<tr>
<td>GATA2P2</td>
<td>ATGAGAAGAGGGCGCTGC</td>
<td>TCCTGGGTTGATAGGTCAG</td>
</tr>
<tr>
<td>Bcl11a</td>
<td>GAGAGAGAAGGACGAGGAAAATG</td>
<td>CTGAGGCAAGGCCGCAAGGAAA</td>
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<tr>
<td>MERTK</td>
<td>TAGGAGAGGAGGAGAAGGCA</td>
<td>AAGAGAAGGGCTGAGAGGAGG</td>
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<td>ELOVL2</td>
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</tr>
<tr>
<td>TP52</td>
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<tr>
<td>WISP2</td>
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<tr>
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<td>v-myc</td>
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<td>CXCL1</td>
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<tr>
<td>CXCL3</td>
<td>CAGAGGAGGGCTGGTTGGA</td>
<td>GAGGAGGCTGAGGAGGAGG</td>
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</table>

Table 1. DNA primers used in the current study

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 deparaffinized 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
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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 immunoreactivity 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 immunoreactivity 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 transected 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.
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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 chordoma.

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.
Brachyury confers chordoma chemoresistance via CA9

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, RASdxmsI1, GATABP2, and MERTK was seen in the PCH1-TOE cell line and expression of CA9, NRARP, and RASdxmsI1 was increased in the U2OS-TOE cell line (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.
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

Table 2. The immunostaining score of CA9 and Brachyury expression

<table>
<thead>
<tr>
<th>Brachyury expression score</th>
<th>CA9 expression score</th>
<th>Total</th>
<th>P</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 (n=7)</td>
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<td>2 (n=14)</td>
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<tr>
<td>-</td>
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<tr>
<td>++</td>
<td>2</td>
<td>6</td>
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<td>1</td>
<td>2</td>
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<td>8</td>
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<tr>
<td>++++</td>
<td>0</td>
<td>2</td>
<td>3</td>
<td>6</td>
</tr>
</tbody>
</table>

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.
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 chemoresistance 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 Brachury-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 chemotherapies of cancers, including chordoma. Further studies are required to define the mechanisms underlying CA9 involvement in chordoma chemoresistance and its value in chordoma therapy.

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Disclosure of conflict of interest

None.

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References


Brachyury confers chordoma chemoresistance via CA9

**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 knockdown 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.