Chordoma and its embryonic determinants

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Abstract

Background. Chordomas are rare malignant tumors occurring along the axial skeleton that originate from notochordal remnants. It is hypothesized that embryonic factors are involved in their aetiology. In the present study, four such factors are of interest: EVX1, which mediates posterior patterning of the embryo and is found in prostate. Wnt3a, localized to the placenta, is involved in different proliferation signaling pathways. Twist is a mediator of the epithelial-mesenchymal transition (EMT) and is expressed in the neuroblastoma cell line SHSY 5Y. Finally, brachyury is a crucial EMT mediator and involved in cell cycle regulation.

Research question. The research question is tripartite. (I) It is the aim to obtain positive control tissue for validation of embryonic primers for the EVX1, Wnt3a and Twist genes. (II) Second, expression of brachyury mRNA in cultured chordoma cells will be assessed in order to validate the cell line. (III) Last, it will be investigated whether fibroblast conditioned cell culturing medium exerts a positive effect on chordoma proliferation in vitro.

Materials and methods. (I) Placenta and prostate samples were obtained from patients in the Maastricht University Hospital. mRNA was isolated from prostate, placenta and SHSY 5Y cells and cDNA of the genes of interest was generated after which PCR and gel electrophoresis was employed to yield amplicon bands. (II) Chordoma cells were cultured and brachyury mRNA expression was assessed by PCR assay. (III) Cultured chordoma cells were grown in enriched DMEM or in enriched DMEM containing 33% fibroblast conditioned medium and proliferation was assessed.

Results. (I) Both EVX1 and Twist are positive in prostate and SHSY 5Y respectively. Wnt3a is not positive in placenta. (II) Brachyury expression is either strongly downregulated or not expressed in two slowly growing chordoma cell cultures. (III) First data indicate enriched DMEM containing 33% fibroblast conditioned medium does not stimulate chordoma proliferation in vitro as compared to enriched DMEM without fibroblast conditioned medium.

Conclusion. (I) Primers are validated for EVX1 and Twist. Placenta and SHSY 5Y can be used as positive control tissue in PCR studies for these genes. (II) Cultured cells no longer express brachyury and the cell lines are not validated for brachyury expression. (III) No apparent difference in growth is present between the enriched DMEM culture and the culture supplemented with 33% fibroblast conditioned medium.

Keywords

Chordoma, notochord, brachyury, primer validation, EVX1, Wnt3a, Twist, cell culture, fibroblast conditioned medium

Introduction

During embryogenesis the notochord is essential for the development of the cranio-caudal axis. As an embryonic signaling center, the notochord guides the formation of the spine (1). During early childhood the notochordal tissue has usually fully regressed (2). However, in certain cases, notochordal remnants remain present within the skull base (most notably the clivus), sacrum and mobile spine. In even rarer cases, these notochordal remnants undergo malignant transformation and turn into a chordoma: a type of aggressive bone tumor with characteristics of both epithelial and mesenchymal tissue (3)particularly with respect to differentially expressed genes involved in the primary origin of chordoma. In this study, therefore, we compared the transcriptional expression profile of one sacral chordoma recurrence, two chordoma cell lines (U-CH1 and U-CH2. Chordomas are rare tumors with an incidence of 0,8 per 1.000.000 per year and a slight male predominance (4,5)a rare tumor arising from notochordal remnants, has been described to date only by single-institution case series or small population-based surveys. METHODS: We used data from the Surveillance, Epidemiology, and End Results (SEER.

One specific diagnostic marker that is expressed virtually ubiquitously in chordomas is the transcription factor brachyury, transcribed from the *T* gene. Brachyury seems to be a key player in the malignant transformation of notochord and is often overexpressed (3) particularly with respect to differentially expressed genes involved in the primary origin of chordoma. In this study, therefore, we compared the transcriptional expression profile of one sacral chordoma recurrence, two chordoma cell lines (U-CH1 and U-CH2. From a functional point of view, the brachyury dimer is involved in regulating different stem cell genes (6) and has also been implicated as a putative mediator of epithelial-mesenchymal transition (EMT) in human carcinomas (7,8).

Next to brachyury, other embryonic factors may be involved in the tumorigenesis of chordoma. The present research aims to gain further insights into the tumor biology

of chordoma by assessing the expression of three such factors: EVX1, Wnt3a and Twist. EVX1 is an embryonic factor previously implied in the specification of posterior positional information within the embryo (9) and is often involved in cancer. Wnt3a induces proliferation through the ERK and Wnt/β-catenin signaling pathways and is further involved in development and cell growth (10). Furthermore, Wnt3a is a downstream target under transcriptional control of brachyury (11)Brachyury or Tbx6, also lack paraxial mesoderm. Here we show that Brachyury is specifically down-regulated in Wnt3a mutants in cells fated to form paraxial mesoderm. Transgenic analysis of the T promoter identifies T (Brachyury, which is relevant in the context of chordoma biology. Finally, Twist has been called "a master regulator of morphogenesis" (12) but has also shown to be involved in several malignancy promoting pathways.

The present study aims to answer three research questions. (I) First, it is the goal to find suitable primers and positive control tissue for EVX1, Wnt3a and Twist. By using tissue obtained from the University Hospital of Maastricht, expression of these genes can be assessed. Before testing for these factors in the chordoma samples, polymerase chain reaction (PCR) primers need to be validated using positive control tissue. Based on literature research it is hypothesized that benign hyperplastic prostate can be useful as a positive control for assessing EVX1 expression (13), placenta can be used for Wnt3a (14) and the neuroblastoma-derived SHSY 5Y cell line may be used for Twist (15). (II) Second, primary chordoma cell cultures will be tested for the diagnostic marker brachyury. (III) Third, we want to evaluate whether fibroblast conditioned medium might enhance the chordoma cells' proliferative capacities.

Material and methods

In order to assess expression of important genes within the pathological gene regulatory networks in the pathogenesis of chordoma, several techniques were employed. Chordoma cells, as well as positive control tissue for PCR assays were necessary for the experiments. Chordoma cells were cultured and brachyury expression was assessed.

Primer validation

For the Wnt3a gene, placental tissue was obtained from a consenting 32 year old female undergoing a planned ceasarian section. For EVX1, benign hyperplastic prostate was obtained from from a consenting 62 year old male undergoing a transuretrhal prostatectomy. Both tissues were flash-frozen in liquid nitrogen to preserve RNA. Tissues were subsequently homogenized using a Mini-Beadbeater-16 (Biospec Products, USA). mRNA was extracted with the RNeasy Plus Mini Kit (Quiagen, USA) from both tissue

homogenates and the SHSY 5Y cell line according to the manufacturer's instructions. cDNA was generated by reverse transcription PCR (RT-PCR) using the RevertAid First Strand cDNA synthesis Kit (Thermo Scientific, USA) according to the manufacturer's instructions. Polymerase chain reactions (PCRs) were performed using primers for the three genes of interest. NCBI's Primer Blast tool was used to create specific exon-spanning primers. All primers were ordered from Sigma-Aldrich (USA). PCR conditions were as follows: 5 minutes at 94 °C followed by 40 cycles of 15 seconds at 94 °C and 1 minute at 60 °C. Inactivation of the polymerase was achieved by heating the sample to 72 °C during 5 minutes. 2% agarose gel electrophoresis at 100V was performed to visualize the PCR products in all PCR assays.

Chordoma cell culture brachyury expression

Two chordoma cell lines designated CH1 and CH2 were available at Maastricht University. They were cultured using Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA) enriched with 10% Fetal Calf Serum (Gibco, USA), 1% penicillin / streptomycin (Gibco, USA), 1% L-glutamine (Gibco, USA) and 1% Insulin Transferrin Selenium supplement (Gibco, USA) (further referred to as: enriched DMEM). The cells were incubated (Sanyo MCO-17AIC, USA) at 37 °C and 5% CO_2. Cultures were propagated to new culturing flasks at 80% confluence. mRNA was isolated from cells of both the CH1 and CH2 culture with the RNeasy mini kit (Quiagen, USA) and brachyury cDNA was generated using the RevertAid First Strand cDNA synthesis Kit (Thermo Scientific, USA) according to the manufacturer's instructions. PCR and gel electrophoresis conditions were as previously described.

Fibroblast conditioned medium and chordoma cell proliferation

We aimed to stimulate a subculture of the chordoma cells by exposing them to enriched DMEM containing 33% fibroblast conditioned medium (FCM). FCM was generated using enriched DMEM cell culture medium further enriched with the excretory products from fibroblasts grown in them. Fibroblasts were then removed from the medium by centrifuging and the medium was subsequently transferred to the chordoma cells.

Results

Primer validation

For the three genes of interest gel electrophoresis yielded separated amplicons. The amplicon length of EVX1 is 74 bp. The EVX1 primer used on the benign hyperplastic prostate is valid and is able to successfully replicate the desired amplicon (figure 1a) the no-template control (NTC) does not show any signal at 74 bp. For the Twist gene (figure 1b), a clear band

is visible at 100 bp in the SHSY 5Y lane indicating that these neuroblastoma cells can also be used as a positive control with this primer. For Wnt3a, the two sets of primers used both yield negative results as no signal at 74 or 100 bp respectively, is obtained (figure 1c, d). Therefore, both sets of used Wnt3a primers are not validated for testing Wnt3a expression in chordoma.

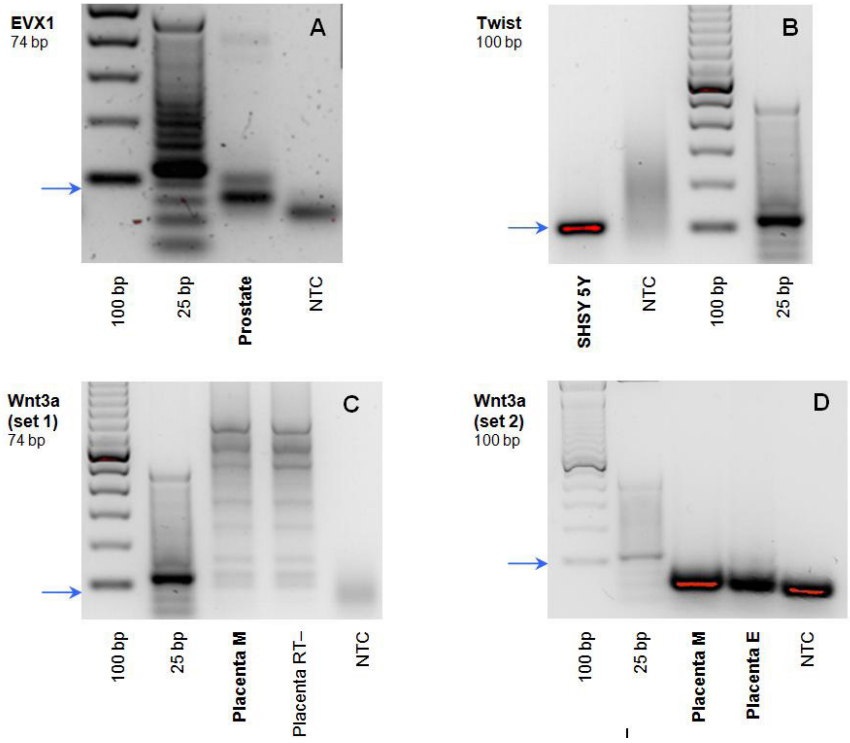

Figure 1. Results of the agarose gel DNA separation for the three genes of interest. EVX1 (A) shows a band at 74 bp and is thus positive in prostate tissue. Twist (B) has an amplicon length of 100 bp and is positive in the SHSY 5Y cell line. Wnt3a (1st primer set: C; 2nd primer set: D) is negative in both the maternal side of the placenta (Placenta M) and the embryonic side (Placenta E). No template control (NTC) is negative in all assays. 100 bp and 25 bp lanes contain DNA ladders of 100 or 25 bp respectively. Blue arrow indicates (expected) location of amplicon.

Chordoma cell culture brachyury expression

Gel electrophoresis of both cultured chordoma cell populations (CH1, CH2) shows a downregulation of brachyury mRNA expression (figure 2). The CH1 cultured cells are

derived from the original Chordoma tissue 1 sample, and the CH2 cells from the Chordoma tissue 2 sample. When comparing CH1 to the tissue from which it was derived, no band is visible at 120 bp in the CH1 lane. Thus CH1 no longer expresses brachyury. Similarly, the CH2 cell culture is characterized by marked downregulation of brachyury. In contrast to the CH1 cell culture, some brachyury expression is still present within the CH2 culture, but this level is lower compared to the Chordoma tissue 2 sample from which CH2 was created. Every sample lane shows (at least to some extent) primer dimer formation as evidenced by the bands at the bottom in these lanes.

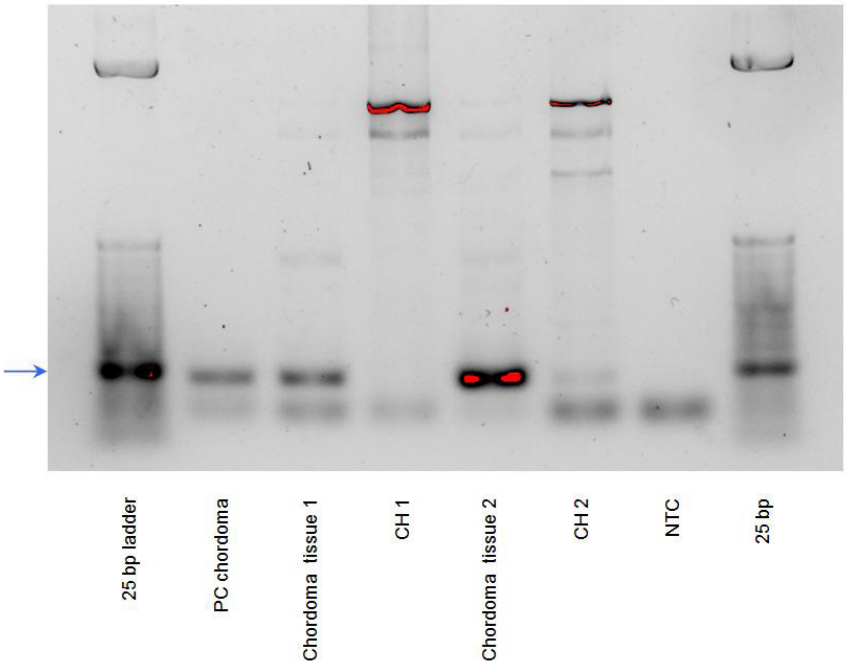

Figure 2. Agarose gel with brachyury amplicons at 120 bp. Positive control (PC chordoma) is known to express brachyury. Chondrosarcoma tissue 1 and 2 both express brachyury. The cultured cells derived from these tissues only expresses brachyury weakly (CH2) or not at all (CH1). No template control (NTC) is negative for brachyury expression. 25 bp lanes contain a 25 bp DNA ladder. Blue arrow indicates (expected) location of amplicon

Fibroblast conditioned medium and chordoma cell proliferation

Microscopically, there seems to be no qualitative evidence that enriched DMEM with 33%

fibroblast conditioned medium induces more proliferation than enriched DMEM only. Using a cell counting chamber it was determined that the CH1 culture has a cell density of 20 cells/µL and the CH2 culture contains 18 cells/µL. However, if such few cells are present, using a counting chamber will not give a reliable estimate of the actual cell content. Furthermore single time point measurements are not able to provide sufficient data for extensive statistical growth and proliferation analysis.

Discussion / Conclusion

For the study of embryonic factors in chordoma appropriate and functional primers need to be created. In order to verify the ability of primers to detect the genes of interest, positive control tissues expressing said genes is necessary. In the present study, three embryonic genes are of interest: EVX1, Wnt3a and Twist. The EVX1 gene is often involved in the posterior patterning of the embryo and has been shown to be modified in certain cancers (9). Expression of EVX1 is high in prostate (13). Wnt3a is part of both the canonical Wnt/β-catenin and the ERK pathway and is therefore an important mediator of proliferation (10). According to literature Wnt3a is mainly expressed in placental tissue (14). The process of epithelial-to-mesenchymal transition is in part reliant on the transcription factor Twist. This process is not only pivotal for successful embryonic development, but is also often considered a metastasis promoting phenomenon in various cancers (16,17). According to literature, Twist expression is high in the neuroblastoma derived SHSY 5Y cell line (15). The primers for both EVX1 and Twist are successful at generating amplicons of the desired genes in benign hyperplastic prostate and SHSY 5Y cells respectively. These tissues can therefore be used as a positive control in gene expression studies. However, the two different primer sets used to amplify the Wnt3a gene in the placental tissue failed to do so. This can be caused by two phenomena. Either the placenta does not contain the Wnt3a (contrary to the literature; references (14,18)), or both sets of created primers are not able to successfully amplify the target sequence. Henceforth these primers are not yet validated and new control tissue will be required to reliably assess the ability of these primers to detect Wnt3a DNA. Optimization of the PCR conditions might also be considered. However, this usually increases amplicon yield, and it is unlikely that variations in temperature and duration of PCR phases will be able to induce the formation of any amplicon, whereas in the used assays, there is none.

The second research question concerns the expression of brachyury in cultured chordoma cells. The PCR assay has shown that brachyury expression is severely diminished in the cultured samples. It can be debated whether these cells are still to be considered true chordoma cells. Chordoma cells tend not to thrive in vitro and proliferate slowly as evidenced

by the limited number of commercially available cell lines (19,20). The CH1 and CH2 cells are no different in that respect. It can be hypothesized that if these cells were to regain brachyury expression, their proliferative capacity would increase since it has been shown that loss of brachyury in chordoma cultures induces growth arrest and senescence (21–23)
Finally, assessing the difference in proliferation between cultured chordoma cells with enriched DMEM and enriched DMEM containing 33% FCM showed no apparent visual difference. However, in order to reliably conclude that the FCM supplementation does indeed have no effect, the proliferation pattern of both cultures needs to be assessed over longer time. Only then repeated cell counting can provide input for statistical calculation and proliferation curves can be generated. From the initial data however, it seems that FCM addition does not contribute to increased proliferation in cultured chordoma cells. There may be different reasons to account for this fact. The chordoma cells might have differentiated into a cell type that phenotypically resembles the initial chordoma, but on a transcriptomics level may bear less resemblance. It is possible that FCM could prevent this differentiation process from occurring if the chordoma cells are exposed to it from the start of the culturing process, rather than adding it later after differentiation processes might already have been initiated. Second, it is possible that the absence of a proliferation increase is the result of the lower nutritional value of the medium supplemented with 33% FCM. Generation of this medium required exposing enriched DMEM to a fibroblast culture. Fibroblast born excretory products are then secreted into the medium, but nutrients in the medium are concurrently consumed by the fibroblasts. This yields a medium with higher levels of growth factors but diminished nutrients.
In terms of embryonic genes, the discussion has focused on EVX1, Wnt3a and Twist hitherto. These are not the only embryonically relevant transcription factors involved in notochord and possibly chordoma development. It is therefore pivotal that more research is directed at elucidating the (embryonic) gene regulatory mechanisms underlying chordoma biology.

Role of the student

At the time of the internship (April – July 2014), Cas Vanderheijden was an undergraduate student in BioMedical Sciences working under the supervision of Prof. dr. Y. Temel and drs. R. Santegoeds at the department of Neurosurgery and the department of Psychiatry and Neuropsychology, part of the Maastricht University Medical Center (MUMC+). The topic was proposed by the supervisors. After agreeing on the experimental designs, experiments were carried out by the student. Analyzing the results, formulating conclusions and writing the thesis was done by the student as well.

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