The Oncogenic IncRNA HOTAIR is not Expressed in the ATRT-MYC-derived Cell Line BT12

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ABSTRACT

Atypical teratoid Rhabdoid tumor (ATRT) is a highly aggressive pediatric tumor of the brain and spinal cord and is one of the most lethal neoplasms of early neonatal life, with a mortality rate exceeding 75%. Almost 95% of ATRT carry a biallelic SMARCB1 gene mutation. ATRT-MYC subgroup is distinguished by elevated levels of HOX Transcript Antisense Intergenic RNA, or HOTAIR, long non-coding RNA (LncRNA). We hypothesize that HOTAIR may play an epigenetic role in ATRT onset of tumorigenesis. In this study, we investigated the existence of potential interaction between the SMARCB1 and the HOTAIR in the non-tumor HEK-293 line, harboring a wild-type SMARCB1. First, the levels of HOTAIR in the BT-12 cell line were assessed using quantitative real-time PCR (qPCR) relative to the non-tumor HEK-293 line, harboring a wild-type SMARCB1, as a calibrator. In contrast to previous reports, our results showed that the HOTAIR expression level was below the detection limit of the qPCR assay, with a quantification cycle (Cq) of ≥ 40 cycles in the BT-12 cell line. Silencing of HOTAIR in HEK-293 cells using antisense oligonucleotides resulted in a significant 32% reduction in wound healing compared to mock-transfected cells.

Keywords
Atypical teratoid/rhabdoid tumor, Pediatric tumors, Brain, Spinal cord.

Introduction
Atypical teratoid/rhabdoid tumor (ATRT) is a rare aggressive pediatric malignancy that affect the brain and spinal cord with a relative 5-year survival rate of only 32.2% [1,2]. ATRTs have a very simple cancer genome, with the predominant, and frequently the only, recurrent molecular hallmark, being a biallelic mutation of the SMARCB1, which encodes a core subunit protein of the SWI/SNF chromatin-remodeling complex [3,4].

The simple genome however contradicts the molecular heterogeneity shown in ATRT cohorts [5]. ATRTs comprise three molecular subtypes that differ in their global level of gene expression, as well as variable degrees of DNA methylation and, are found in distinct anatomical locations [6]. The three main molecular subgroups of ATRTs, for which a consensus was reached to name them ATRT-TYR, ATRT-SHH, and ATRT-MYC [7].

SMARCB1, also named INI1 or SNF5 [8], is a tumor suppressor gene located on chromosome 22q11.2 [9]. The initiation and progression of aggressive cancers caused by SMARCB1 loss may occur epigenetically via disturbance of transcriptional regulation rather than destabilizing the genome through the accumulation of mutations [10].

BT-12 is a ATRT cell line that has been allocated to MYC subgroup, that, generally, express elevated levels of HOTAIR [5]. HOTAIR lncRNA interacts with SMARCB1 subunit protein in clear cell renal cell carcinoma (ccRCC) [11].

Long noncoding RNAs (lncRNAs) are transcripts of 200 nucleotides (nt) or more lacking an open reading frame (ORF) that have been
reported to regulate transcription via recruitment of chromatin modifiers or bridging distal enhancer elements to gene promoters [12,13]. HOX transcript antisense RNA (HOTAIR) is a lncRNA with 6 exons and more than 2000 nucleotides [14]. HOTAIR is an oncogenic factor and plays a key role in the initiation, progression, and promotion of malignancy in different types of cancer [15]. Transcriptome analysis of 20 pediatric ATRT using nanoString platform revealed high expression of HOTAIR [16].

In this study, we investigated whether a possible link exists between SMARCB1 loss in BT-12 ATRT cell line and the reported high HOTAIR expression in ATRT and the potential role of HOTAIR as an epigenetic regulator between loss of function of SMARCB1 and the onset of tumorigenesis. We used real-time quantitative PCR (qPCR) to assess the expression level of HOTAIR in the authenticated ATRT-derived cell line, BT-12, compared to the non-tumor human line HEK293 hosting a wildtype SMARCB1. We also used customized small interfering RNAs (siRNAs) to silence HOTAIR in HEK-293 in order to explore the influence of such knock down on SMARCB1 mRNA expression in this cell line or whether the loss of SMARCB1 might have influenced the expression of HOTAIR. We document that the HOTAIR expression level in BT-12 is below the qPCR limit of detection. This result suggests that HOTAIR plays no oncogenic role in the BT-12 cell line.

**Methods**

**Materials**

Roswell Park Memorial Institute (RPMI), Dulbecco’s Modified Eagle’s Medium (DMEM), and Phosphate Buffered Saline (PBS) were purchased from Lonza Bioscience, Absolute Ethanol was purchased from Honeywell, DEPC-treated water and Nuclease free water were purchased from Thermo Fisher Scientific, Chloroform was purchased from Carl Roth.

**Cell culture**

ATRT cell line, BT-12, was a gift from Peter J. Houghton. The human embryonic kidney cell line, HEK-293, was purchased from The American Type Culture Collection. BT-12 cells were grown in RPMI medium and supplemented with 10% fetal bovine serum (FBS, Gibco). HEK-293 cells were cultivated in a DMEM medium supplemented with 5% FBS. For both lines were supplemented with 1% penicillin/streptomycin (Gibco). The cell lines were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO2.

**Total RNA extraction and quantification**

Total RNA was extracted from both BT-12 and HEK-293 cell lines using the TRIzol reagent (Thermo Fisher Scientific), according to the manufacturer’s instructions and was quantified using a Qubit® assay kit according to the manufacturer’s instructions (Thermo Fisher Scientific).

**Reverse transcription and real-time PCR**

cDNA was synthesized using a SuperScript® III First-Strand Synthesis System kit (Thermo Fisher Scientific). Quantitative real-time PCR (qPCR) analysis was performed in triplicate using TaqMan Fast Advanced PCR Master Mix in customized assays (The assay ID for SMARCB1 primers: Hs00992516_m1, for HOTAIR: Hs03296631_m1, and for GAPDH: Hs00266705_g1) on a StepOnePlus™ Real-Time PCR System according to the manufacturer’s protocol (Thermo Fisher Scientific). The exact binding sites of the primers and TaqMan probes are shown in Supplementary Figure 1. The PCR program was as follows: denaturation at 95°C for 2 minutes and 40 cycles of amplification consisting of denaturation at 95°C for 10 seconds, annealing and extending at 60°C for 20 seconds. The Human GAPDH level was used as an internal control. Relative expression was assessed using the 2^△△CT method [17].

**HOTAIR knockdown in HEK-293 cell line**

Small interfering RNAs (siRNAs) specifically targeting HOTAIR (siHOTAIR), siRNA specific for GAPDH (siGAPDH), and its negative control (siNC), were purchased from Thermo Fisher Scientific. All these siRNAs were transfected into the HEK-293 cell line using Lipofectamine RNAiMAX reagent (Thermo Scientific), referring to the instructions of the manufacturer. The assay ID for siHOTAIR: n272221.

**Wound Healing Assay**

HEK-293 cells were cultured in the 6-well plate and a straight line was drawn with a 10-μL sterile pipette tip. The repair was observed under a microscope at 0 and 24 hours. The wound area (scratch area) was calculated using an online tool, [https://www.sketchandcalc.com/](https://www.sketchandcalc.com/).

The rate of wound healing was calculated according to the formula:

\[ \text{Rate of wound healing} = \frac{\text{wound area at time 0 hour} - \text{wound area at time 24 hours}}{\text{wound area at time 0 hour}} \times 100 \]

**Results**

**qPCR results for HOTAIR expression levels in the BT-12 cell line.**

The qPCR experiments revealed two unexpected results. First, in BT-12 the SMARCB1 is expressed on the mRNA level albeit at a very low concentration. Second, compared to HEK-293, the HOTAIR level is below the detection level in contrast to the reported upregulation in the ATRT-MYC subgroup (Figure 1).

**Figure 1**: SMARCB1, and HOTAIR Fold gene expression in BT-12 relative to HEK-293 (calibrator).
Silencing of HOTAIR in HEK-293 cells.

Calculations using the comparative (Cq) method show successful HOTAIR siRNA treatment of HEK-293 relative to HEK-293 treated by negative control siRNA (non-targeting siRNA control) with a knockdown efficiency of 88%, approximately (Figure 2).

**Figure 2:** HOTAIR Fold gene expression in HEK-293 treated by HOTAIR siRNA relative to HEK-293 transfected with a negative control siRNA (calibrator).

Wound healing assay

The rate of wound healing i.e., % Closure of the wound in the siHOTAIR, siNC groups were 54%, and 79% respectively (see Figure 3,4). The results indicate that HOTAIR knockdown in HEK-293 cells decreased cell migration rate by 32% compared to the negative control group.

**Figure 3:** Negative control experiment using non targeting siRNA in wound healing (scratch) assay for HEK-293 cells.

**Figure 4:** Positive control experiment using GAPDH siRNA in wound healing (scratch) assay for HEK-293 cells.

Discussion

The simple genomes of ATRT tumors imply the involvement of an epigenetic dimension of regulation accomplished by chromatin condensation, DNA methylation at promoter CpG islands and/or noncoding RNAs.

**SMARCB1** forms a core subunit of the SWI/SNF chromatin remodeling complex, which is a critical regulator of human embryonic development. This subunit is frequently described as a tumor suppressor that abolishes super-enhancers controlling hESC lineage determination [18]. The nature of this interaction, however, is not clearly understood. The reported upregulation of lncRNAs in ATRT encouraged us to hypothesize that noncoding RNAs, such as HOTAIR, might mediate the SMARCB1-super-enhancer interaction. This suppressive level of regulation antagonizes the oncogenic MYC protein, also reported to be regulated by SMARCB1 [19]. Thus, the loss of SMARCB1 function can drive malignancy in ATRT.

Our qPCR results showed that SMARCB1 is low level of mRNA expressed in BT-12. This comes in agreement with an earlier observation by (Biegel et al.) who reported the expression of SMARCB1 in three out of seven primary rhabdoid tumors [20].

The oligonucleotides of the SMARCB1 assay used in this study (Hs00992516_m1; ThermoFisher Scientific) target the 5’-region of the gene with the probe spanning exons 1 and 2. Thus, in BT-12, at least the initial part of SMARCB1 mRNA is being transcribed or carries a nonsense mutation that apparently did not affect oligo binding.

Chakravadhanula et al. studied the expression of the HOX and HOTAIR genes in pediatric tumors (20 ATRTs, 10 ependymomas, 10 medulloblastomas, 6 glioblastoma multiforme, and 9 juvenile pilocytic astrocytomas (JPAs) using nanoString technology. They report high expression level of the HOXC and HOTAIR genes [16]. Our qPCR results in BT-12, however, contradict this finding; in this cell line, HOTAIR expression was below the detection limits of the assay.

The BT-12 cell line has been allocated to the ATRT-MYC molecular subgroup of rhabdoid tumors by several authors [5,6]. Members of this subgroup typically exhibit high expression levels of MYC, lack tyrosinase signature, and show an elevated HOX gene expression. This could align with our results that BT-12 also lacks the HOTAIR that is typically upregulated in ATRT-MYC.

Our study however, does not negate the possibility of HOTAIR interacting with SMARCB1 in other cell lines allocated to ATRT-MYC or in other tumors in general. For example, (Sumida et al.) performed RNA immunoprecipitation (RIP) assays in renal cell carcinoma and found that HOTAIR binds to both SMARCB1 and ARID1A [21].

siRNA-mediated knockdown of HOTAIR in HEK-293 cells reduced cell migration rate by 32% compared to the negative control.
siRNA group, the reason is that HOTAIR causes dysregulation in the expression of proteins involved in diverse cellular components, including the cytoskeleton and the respiratory chain. (Zheng et al.) did functional studies on vimentin (VIM), a key protein involved in maintaining the integrity of the cytoskeleton and cell shape, revealed that HOTAIR exerts its effects on migration and invasion of HeLa cells, at least in part, through the regulation of VIM expression [22]. Also, HOTAIR can promote cell migration and invasion by regulating Megakaryoblastic leukemia 1 (MKL1) via inhibition of miR206 expression in HeLa cells [23].

References

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