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Epigenetic genome-wide analysis identifies *BEX1* as a candidate tumour suppressor gene in paediatric intracranial ependymoma

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ABSTRACT

Promoter hypermethylation and transcriptional silencing is a common epigenetic mechanism of gene inactivation in cancer. To identify targets of epigenetic silencing in paediatric intracranial ependymoma, we used a pharmacological unmasking approach through treatment of 3 ependymoma short-term cell cultures with the demethylating agent 5-Aza-2′-deoxycytidine followed by global expression microarray analysis. We identified 55 candidate epigenetically silenced genes, which are involved in the regulation of apoptosis, *Wnt* signalling, *p*53 and cell differentiation. The methylation status of 26 of these genes was further determined by combined bisulfite restriction analysis (COBRA) and genomic sequencing in a cohort of 40 ependymoma samples. The most frequently methylated genes were *BEX1* (27/40 cases), *BAI2* (20/40), *CCND2* (18/40), and *CDKN2A* (14/40). A high correlation between promoter hypermethylation and decreased gene expression levels was established by real-time quantitative PCR, suggesting the involvement of these genes in ependymoma tumourigenesis. Furthermore, ectopic expression of brain-expressed X-linked 1 (*BEX1*) in paediatric ependymoma short-term cell cultures significantly suppressed cell proliferation and colony formation. These data suggest that promoter hypermethylation contributes to silencing of target genes in paediatric intracranial ependymoma. Epigenetic inactivation of *BEX1* supports its role as a candidate tumour suppressor gene in intracranial ependymoma, and a potential target for novel therapies for ependymoma in children.

1. Introduction

Ependymoma is the third most common primary brain tumour in children, following astrocytomas and medulloblastomas, accounting for 5–11% of all brain tumours in the paediatric population [1,2]. Five-year survival ranges from 53% to 75% depending on age at diagnosis and extent of surgical resection, with patients <2 years of age or with residual tumour after surgery having significantly poorer outcome [3,4].

Recently, the application of high-resolution microarray-based platforms for the detection of changes in DNA copy numbers and gene expression has facilitated our understanding of the genetic events underlying ependymoma pathogenesis. Gain of chromosome 1q has been shown to be the most common genomic imbalance in paediatric ependymoma using meta-analysis of comparative genomic hybridisation (CGH) data [5]. Indeed, gain of chromosome 1q and the region-specific amplicon 1q25 have been shown to be adverse prognostic biomarkers in paediatric intracranial ependymoma using metaphase CGH analysis [6], array CGH [7,8], quantitative polymerase chain reaction (Q-PCR) [9] and single-nucleotide polymorphism arrays [10]. Furthermore, microarray profiling revealed differences in DNA copy numbers and gene expression signatures, which were associated with tumour location and grade [5,8,11]. Loss of 9p, for example, has been shown to occur preferentially in paediatric supratentorial ependymoma [5] while gain of 1q and/or homozygous deletion of *CDKN2A* has been demonstrated to be an independent prognostic factor of poor prognosis in anaplastic intracranial ependymoma [8].

Epigenetic alterations, such as DNA methylation, have also been shown to be an important mechanism for transcriptional inactivation of tumour suppressor genes (TSGs) [12]. To date, a limited number of studies have shown promoter hypermethylation of a

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number of genes, including *HIC1*, *RASSF1A*, *CDKN2A*, *CDKN2B*, *MCJ MGMT*, *p73* and *TRAIL* apoptosis pathway-related genes (*CASP8*, *TFRSF10C* and *TFRSF10D*), in paediatric ependymoma [13–17]. Recent evidence has also suggested extensive hypermethylation of TSGs as an important mechanism in the pathogenesis of spinal and supratentorial paediatric ependymoma using a cancer methylation array [18].

Pharmacological unmasking of epigenetic alterations with the demethylating agent 5-Aza-2′-deoxycytidine (5-Aza-dC) coupled with microarray analysis is a powerful tool that has been successfully used in the identification of key methylated TSGs and regulated pathways in many tumours [19,20], including malignant brain tumours [21]. In the present study, using a combined approach of pharmacological inhibition of DNA methylation through treatment of ependymoma short-term cell cultures with 5-Aza-dC followed by global gene expression microarray analysis, we have identified candidate genes of epigenetic silencing in paediatric intracranial ependymoma. The methylation status of 26 target genes was determined by combined bisulfite restriction analysis (COBRA) and bisulfite sequencing in a cohort of 40 ependymoma tumours. Promoter hypermethylation of four genes, *BEX1*, *CCND2*, *BAI2* and *CDKN2A*, was strongly correlated with decreased expression levels of these genes as it was established by real-time quantitative PCR. In addition, the role of one of these candidate genes, brain-expressed X-linked 1 (*BEX1*), was assessed for its functional role as a candidate tumour suppressor in paediatric ependymoma *in vitro*. Our results showed that exogenous expression of *BEX1* in ependymoma short-term cell cultures significantly inhibited cell proliferation and colony formation.

2. Materials and methods

2.1. Tissue samples and cell cultures

Forty tumour samples were obtained with informed consent from 38 patients, the clinical features of which have been reported previously [9]. All tumours were classified histologically and graded according to the WHO classification [22] by 2 neuropathologists (Great Ormond Street Hospital for Children, UK). They comprised 26 ependymoma (grade II) and 14 anaplastic ependymoma (grade III), 27 of which were primary and 13 recurrent tumours, including 2 patients with multiple resections (primary/recurrent pairs). Of the 40 tumour samples, 30 were fresh frozen biopsy samples, which were collected directly by surgery and stored in liquid nitrogen until DNA and total RNA was extracted. Samples were directly adjacent to tumour tissue processed for routine histological evaluation and tissues containing more than 80% tumour cells were selected for DNA and RNA extractions.

Ten short-term cell cultures were prepared as described previously [23] and grown in Hams F10 nutrient mix supplemented with 10% selected fetal calf serum (Invitrogen, UK) at 37 °C in a non-CO_2 incubator. The U87-MG glioblastoma multiforme (GBM) cell line was obtained from American Type Culture Collection (Manassas, VA) and maintained under identical conditions.

2.2. 5-Aza-2′-deoxycytidine treatment and expression array analysis

Three short-term ependymoma cell cultures (IN1594, IN1638 and IN3008) were used for whole genome expression analysis following treatment with the demethylating agent 5-Aza-2′-deoxycytidine (5-Aza-dC). Prior to microarray analysis, the optimum dosage of 5-Aza-dC to effect demethylation without inducing cytotoxicity was determined using a standard MTT assay as described below. Demethylation of a panel of 5 genes was confirmed using reverse transcription-polymerase chain reaction (RT-PCR) analysis. For expression array analysis, cells were plated at low passage number (passage 6–8) in T75 cm^2 flasks (1×10^6 cells) in media with 10% fetal calf serum and left overnight to attach. 5-Aza-dC (Sigma–Aldrich, Inc.) was freshly prepared in distilled water and filter sterilized. Cells were treated with 5 μmol/L 5-Aza-dC or a control volume of vehicle (DMSO final concentration of 0.1% v/v; Sigma). Medium and drug or vehicle were replaced every 24 h over a 96-h period.

Total RNA from treated and mock-treated cells was extracted using the RNeasy Mini Kit according to the manufacturer's instructions (Qiagen Ltd., UK). All RNA samples were treated with DNase I (Thermo Scientific). The quality and quantity of each sample was assessed using a NanoDrop® ND2000 UV–Vis spectrophotometer (LabTech International Ltd., UK) and only RNA samples with an A260/A280 ratio $\geqslant$1.8 and an A260/A230 ratio of $\geqslant$2.0 were used.

For microarray analysis, total RNA (5 μg) was reverse transcribed with the MessageAmp™ II-Biotin *Enhanced* Single Round aRNA Amplification Kit following manufacturer's instructions (Ambion, Life Technologies Ltd. UK) and hybridised to the Human Genome U133 Plus 2.0 oligonucleotide array (Affymetrix UK Ltd.). The arrays were washed and stained using the GeneChip Fluidics Station 450 and scanned with the GeneChip Scanner 3000 7G (Affymetrix) according to standard manufacturer protocols. Experiments were performed in duplicate from biological replicates for both treated and untreated cell cultures.

2.3. Microarray data analysis

Raw microarray data from Affymetrix CEL files from each cell culture were initially normalised using the MAS5 (Affymetrix Microarray Suite, version 5.0) algorithm. The raw intensity values were further assessed by the Robust Multi-array Analysis (RMA) as implemented in Bioconductor (http://www.bioconductor.org/). The RMA intensity in log2 scale was generated for each probe set and the perfect-match (PM) intensities per probe set were background-corrected, quantile-normalised and signal intensities for each probe set were summarised using median signal intensity to fit linear model (GeneSpring GX 7.3.1.). Array background, *Q* values, and mean intensities were within acceptable ranges for all arrays. The data sets supporting the results of this article are available in the Gene Expression Omnibus (GEO) according to their guidelines with accession number GSE45437, (http://www.ncbi.nlm.nih.gov/geo/).

Statistical comparisons and data visualisation were performed using GeneSpring GX 7.3.1 (Agilent Technologies). The Cross-Gene Error Model (GeneSpring) was applied on replicate per cell culture per treatment. The "Filter on Fold Change" tool was then applied to select up-regulated genes that showed a difference of $\geqslant$3-fold increase in expression levels in the 5-Aza-dC-treated cultures compared to untreated controls as previously described [21]. Statistically significant genes were identified using 1-Way ANOVA ($P < 0.05$) and a Benjamini and Hochberg False Discovery Rate multiple correction test (GeneSpring).

For significantly enriched epigenetically regulated signalling pathways, the commonly up-regulated genes in treated cell cultures were further analysed by Onto-Tools Pathway-Express (http://vortex.cs.wayne.edu/projects.htm) and PANTHER Classification System (http://www.pantherdb.org/).

2.4. Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA (1 μg) isolated from cells treated with either 5 μM 5-Aza-dC or mock-treated was reverse-transcribed with the QuantiTect cDNA synthesis kit using HotStarTaq DNA Polymerase (Qiagen). Gene primers were designed using Primer3 software v.4.0 (http://frodo.wi.mit.edu/) and are shown in Supplementary Table S1. PCR cycling conditions consisted of an initial activation step at 95 °C for 15 min, followed by 35 cycles of 94 °C denaturing for 30 s, primer annealing at 52–56 °C (gene-dependent) for 30 s, product extension at 72 °C for 30 s, and a final extension at 72 °C for 10 min. The β-actin gene was used as a reference control. Amplification products were visualised on 2.0% agarose gels by ethidium bromide staining.

2.5. Bisulfite modification of DNA, combined bisulfite restriction analysis (COBRA) and direct sequencing

Genomic DNA was extracted from 30 biopsy samples, 10 short-term cell cultures and 15 blood samples from patients with ependymoma using the DNeasy Blood and Tissue Kit following the manufacturer's instructions (Qiagen). The quality and quantity of genomic DNA was assessed using the NanoDrop® ND2000 spectrophotometer.

Bisulfite treatment of DNA (1 μg) was performed using the EZ DNA Methylation Gold Kit as per manufacturer's instructions (Zymo Research, Cambridge, UK). CpG islands upstream of the transcriptional start site of target genes were identified by MethPrimer software (www.urogene.org/methprimer) whilst putative promoter regions were predicted by Genomatix Promoter Inspector software (http://www.genomatix.de/online_help/help_gems/PromoterInspector_help.html).

Bisulfite-modified DNA (2 μl) were amplified in 20 μl volume using specific primers for 26 candidate silenced genes and 1 U HotStarTaq DNA polymerase (Qiagen). Touch-UP gradient PCR was performed as described previously [24]. Amplification products were digested by *Bst*UI (CGCG) and *Taq*I (TCGA) (Fermentas, UK) at 37 °C and 65 °C respectively and visualised on 4.0% agarose gels with ethidium bromide staining. Primers for COBRA analysis and restriction products are provided in Supplementary Table S2.

For direct sequencing, bisulfite-modified DNA was amplified using the primers for COBRA analysis and separated on 3.0% agarose gels. PCR products were purified with PureLinkTM Quick Gel Extraction kit (Invitrogen) and sequenced on an ABI 3730 Sequencer with the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems; University of Cambridge, UK). Sequenced products were analysed with the Sequence Scanner Software v1.0 (Applied Biosystems). DNA from normal bloods and universal methylated DNA (Zymo Research) were used as unmethylated and methylated controls in COBRA analysis and sequencing, both of which were bisulfite-treated as described above.

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