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The Long Non-Coding RNA H19 Drives the Proliferation of Diffuse Intrinsic Pontine Glioma with H3K27 Mutation

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Abstract: Diffuse intrinsic pontine glioma (DIPG) is an incurable paediatric malignancy. Identifying the molecular drivers of DIPG progression is of the utmost importance. Long non-coding RNAs (lncRNAs) represent a large family of disease- and tissue-specific transcripts, whose functions have not yet been elucidated in DIPG. Herein, we studied the oncogenic role of the development-associated H19 lncRNA in DIPG. Bioinformatic analyses of clinical datasets were used to measure the expression of H19 lncRNA in paediatric high-grade gliomas (pedHGGs). The expression and sub-cellular location of H19 lncRNA were validated in DIPG cell lines. Locked nucleic acid antisense oligonucleotides were designed to test the function of H19 in DIPG cells. We found that H19 expression was higher in DIPG vs. normal brain tissue and other pedHGGs. H19 knockdown resulted in decreased cell proliferation and survival in DIPG cells. Mechanistically, H19 buffers let-7 microRNAs, resulting in the up-regulation of oncogenic let-7 target (e.g., SULF2 and OSMR). H19 is the first functionally characterized lncRNA in DIPG and a promising therapeutic candidate for treating this incurable cancer.

Keywords: paediatric glioma; DIPG; diffuse midline glioma; lncRNA; epigenetics; brain cancer

1. Introduction

Malignant brain tumours are a leading cause of death in paediatric patients. Diffuse intrinsic pontine glioma (DIPG) is a type of paediatric high-grade glioma (pedHGG) originating in the brainstem and affecting children with a median age of six to seven years [1,2]. DIPGs are highly infiltrative and belong to the fibrillary astrocytoma family, where lesions are classified as either WHO grade III or IV [3]. At the molecular level, substitution of lysine at the 27 position of the histone 3 locus with methionine (H3K27M) is usually identified in approximately 80% of histologically confirmed DIPGs [4]. H3K27M substitution is identified in approximately 80% of histologically confirmed DIPGs. The World Health Organization has recently classified DIPGs in the broader category of diffuse midline gliomas (DMGs) with a H3K27M mutation [2,5–7]. Another frequent mutation of the H3 locus, GR4R, is more prevalent in pedHGGs located in the cerebral hemispheres [8]. The surgical removal of DIPGs is almost impossible due to their infiltrative nature and their location. Currently, radiotherapy is the standard treatment for this malignancy; despite this treatment, the median survival for DIPG patients is 9–11 months [9,10]. Therefore, novel therapeutic targets are necessary to improve the prognosis of these paediatric patients.
There are approximately 60,000 long non-coding RNAs (lncRNAs) in the human genome; these transcripts are defined as non-coding transcripts longer than 200 nucleotides. Some lncRNAs are highly expressed under pathological conditions and regulate key aspects of tumour progression, invasion, and metastasis [11,12]. Previous studies have identified several lncRNAs associated with DIPG progression [13,14]. However, no lncRNAs have been functionally characterised in this malignancy.

H19 (gene ID: 283120) is an extensively studied lncRNA, whose aberrant expression has been linked to alterations during foetal development [15]. The oncogenic role of H19 has been described in several malignancies, including adult gliomas [16–18]. Mechanistically, H19 expression is triggered by hypoxia; this lncRNA can affect a wide range of hub genes, including microRNAs (miRNAs) and mRNAs [19,20]. However, the function of H19 in pedHGG still remains largely unknown.

Herein, we investigated the clinical relevance of H19 in DIPG cells and tested H19 targeting for halting DIPG proliferation.

2. Results

2.1. H19 Is Up-Regulated in DIPG Tissue

To confirm the expression of H19 lncRNA in DIPG tissue, we analysed open-access clinical datasets of pedHGGs and normal brain tissue. Bioinformatic analyses confirmed that H19 levels are significantly increased in the DIPG tissue of the “Allis-45-custom-ilmnht12v4” dataset (p < 0.01, Figure 1a) whereas an increasing trend was observed in “Paugh-37-MAS5.0-u133p2” (Figure 1b). Microarray data from Paediatric Cbioportal (https://pedcbioportal.kidsfirstdrc.org/, accessed 30 June 2020) demonstrated significantly higher H19 expression in the brainstem than in hemispheric pedHGGs (p < 0.01) (Figure 1c). We then explored the influence of mutations in the H3 genes (H3K27M and WT) on H19 expression; this analysis revealed that H3K27M-bearing pedHGGs express higher levels of H19 compared to pedHGGs bearing the wild-type H3 gene (p < 0.01) (Figure 1d). Histological classification of pedHGG samples showed that DIPG tissues express higher H19 levels than other pedHGGs, including anaplastic astrocytoma and glioblastoma (p < 0.01) (Figure 1e). Overall survival analysis did not identify significant differences between high- and low-H19-expressing DIPG groups in the CBioportal dataset (median survival was 9.8 and 11.1 months, respectively) (Figure 1f). Analysis of a single-cell RNA sequencing dataset from the developing human midbrain showed that H19 is most strongly expressed by oligodendrocyte precursor cells (OPCs), which are thought to be the cells of origin of DIPGs [21,22] (Figure 1g). All of these results indicate that H19 is highly and selectively up-regulated in DIPGs with H3K27M mutation. Hence, we decided to study the cellular function and mechanism of action of this lncRNA in DIPG-H3K27M.

Figure 1. Cont.
Figure 1. H19 is associated with DIPGs (a) Clinical expression data of H19 in the “Allis-45-custom-ilmnht12v4” dataset analysed on the R2 platform for normal brain (n = 10) and DIPG tissues (n = 35) [23,24]. (b) Clinical expression levels of H19 in the “Paugh-37-MAS5.0-a133p2” dataset analysed on the R2 platform for normal brain (n = 2) and DIPG tissues (n = 27). (c) H19 expression in patient samples with pedHGGs located in either hemisphere (n = 107), midline (n = 46), or brainstem (n = 65) analysed with the paediatric Cbioportal platform [25]. (d) H19 expression in patient samples with pedHGGs either non-mutated/WT (n = 118) or carrying a H3K27M mutation (n = 83) analysed with the paediatric Cbioportal platform. (e) Expression of H19 in histologically confirmed DIPG samples (n = 65) or other pedHGGs (n = 153). (f) Kaplan–Meier curve for overall survival time (months) of DIPG patients with either high or low levels of H19 (median expression cut-off). (g) Violin plot showing the Bayesian posterior probability of H19 expression in different cell types during neurodevelopment. Data in (a,d,e) were analysed by an unpaired two-tailed t-test with Welch’s correction. Data in (e) were analysed with one-way ANOVA and Dunnett’s post-hoc test for multiple comparison. All data are shown as mean ± SD. Significant values are **p < 0.01, ***p < 0.001. NS = non-significant.

2.2. H19 Is Required for DIPG Cell Viability but Not Migration

To investigate the functional role of H19, expression of this transcript was analysed in a panel of human DIPG cell lines and normal astrocytes. The malignant cell lines showed higher levels of H19 IncRNA expression compared to normal astrocytes. VUMC-DIPG-A cells expressed the highest levels of H19 (Figure 1a). Biologically, the VUMC-DIPG-A cell line bears the H3.3K27M mutation, whereas SU-DIPG-IV cells carry the H3.1K27M mutation (Supplementary Table S1). Cell fractionation assays demonstrated that H19 IncRNA is ~3-fold enriched in the cytoplasm of both VUMC-DIPG-A and SU-DIPG-IV cells compared to the nucleus (Figure 2b).
Figure 2. H19 is required for DIPG cell proliferation but not migration. (a) Expression (qPCR) of H19 lncRNA in a panel of DIPG cancer cells and a non-neoplastic human astrocyte cell line (n = 3). (b) Subcellular localisation of H19 in VUMC-DIPG-A and SU-DIPG-A cells. Internal control included GAPDH (cytoplasm control) and MALAT1 (nuclear control) (n = 2 and n = 3, respectively). (c) H19 RNA relative expression on LNA-silenced VUMC-DIPG-A cells (LNA1, LNA2, and LNA3) to negative control LNA-silenced VUMC-DIPG-A cells (n = 3). (d) Trypan blue exclusion cell number of VUMC-DIPG-A after 2, 4, and 6 days post-transfection with LNA2 or negative control LNA (n = 3). (e) Representative brightfield image of LNA2- or negative control LNA-transfected VUMC-DIPG-A cells after 2, 4, and 6 days. Magnification 10×. (f) Dose response (0.05 to 500 nM) effect of LNA7 silencing on VUMC-DIPG-A cell number (n = 2). (g) Cell viability-adjusted activity of caspase 3/7 after 4 days of LNA2-mediated H19 knockdown in VUMC-DIPG-A cells (n = 3). (h) Cell number of normal human astrocytes after transfection with either LNA2 (50 nM) or negative control LNA on day 4 (n = 3). (i) Wound healing assay showing wound area ratio closing at 8, 24, and 32 h post-scratch (n = 3). (j) Brightfield representative images of wound healing assay. Magnification 10×. Data on (a) was analysed with a one-way ANOVA followed by Dunnett’s multiple comparison test. Data on (b–d) were analysed with a two-way ANOVA with Sidak’s post-hoc test for multiple comparison. Data are shown as mean ± SD. Significant values were *p < 0.05, **p < 0.01, ***p < 0.001, or ****p < 0.0001.
To understand the cellular function of H19, we performed gene silencing studies on VUMC-DIPG-A cells using locked-nucleic acid (LNA) antisense oligonucleotides. Three LNAs were designed and tested in this cell line. Two days post-transfection, H19 expression vs. the control was 0.47 ± 0.37 with LNA1, 0.16 ± 0.09 with LNA2 (p < 0.05), and 2.04 ± 2.59 with LNA3 (Figure 2c). LNA2 was therefore selected for cell proliferation assays. We found that cell numbers failed to increase in the presence of LNA2, which instead induced a significant and durable reduction in the number of cells on days 4 and 7 (p < 0.01 and p < 0.05, respectively) (Figure 2d). VUMC-DIPG-A cells were also imaged using bright-field microscopy, which showed morphological changes such as detached cells indicative of cell death upon exposure to LNA2 (Figure 2e). We also observed that LNA2 induced a dose-dependent inhibition of DIPG cell numbers, with an estimated IC\textsubscript{50} of 52.4 ± 1.4 nM on day 4, measured by trypan blue exclusion (Figure 2f). In order to determine whether H19 knockdown attenuated cell proliferation by inducing caspase-dependent apoptosis, caspase 3/7 assays were carried out on day 4. H19 silencing via LNA2 triggered a significant increase in caspase 3/7 activity (p < 0.01) (Figure 2g). Notably, H19 knockdown in non-neoplastic astrocytic cells did not significantly affect cell proliferation (Figure 2h). To investigate whether H19 was involved in the migration of VUMC-DIPG-A cells, a wound healing assay was performed 24 h post-transfection. H19 knockdown did not affect the migration of VUMC-DIPG-A cells in this experimental design (Figure 2i,j). Overall, these functional assays suggest that H19 modulates DIPG-H3K27M cell proliferation and apoptosis.

2.3. Let-7a-5p Affects DIPG-H3K27M Cell Proliferation

To determine the mechanism of action behind H19-driven cell proliferation, we compared the transcriptome of H19 knockdown vs. control DIPG cells. RNA sequencing analysis revealed 449 up-regulated and 622 down-regulated mRNAs upon H19 knockdown using LNA2 in VUMC-DIPG-A cells (Figure 3a and Supplementary Table S2). Gene set enrichment analysis revealed that the most up-regulated protein-coding genes were associated with tumour necrosis factor alpha (TNF\textalpha) signalling, apoptosis, or KRAS signalling. Down-regulated genes were associated with epithelial and mesenchymal transition, UV response, or TGF\beta signalling (Figure 3b).

Cytoplasmic lncRNAs often interact with miRNAs. Typically, these lncRNAs bind to complementary miRNAs, thereby displacing the interaction of the miRNA and its mRNAs targets; this results in increased translation of the target mRNAs [26]. We therefore decided to test the hypothesis that H19 exerts its oncogenic function by buffering onco-suppressive miRNAs. As the first step, we searched the TARBASE dataset, and identified 11 miRNAs that are validated targets of H19 (Figure 3c and Supplementary Table S3). Notably, the let-7 miRNA family was highly represented in this list. Hence, we decided to focus further investigation on let-7a-5p, whose direct binding to H19 has been demonstrated experimentally [27]. However, it is still unknown whether let-7a-5p affects DIPG cell proliferation.

To test our hypothesis, we transfected a miRNA mimic of let-7a-5p in DIPG cells. Transient overexpression of let-7a-5p led to a significant reduction in VUMC-DIPG-A cell proliferation five days post-transfection (Figure 3d). Morphologically, let-7a-5p-overexpressing VUMC-DIPG-A cells showed no obvious differences compared to those transfected with the negative control mimic (Figure 3e). These results suggest that let-7a-5p is involved in DIPG-H3K27M cell proliferation.
**Figure 3.** let-7a-5p reduces DIPG-H3K27M cell proliferation and migration. (a) Volcano plot of RNA-seq expression on H19-silenced VUMC-DIPG cells (red dots are \( p < 0.01 \)). (b) Gene set enrichment analysis of up- and down-regulated (log2FC < or >0.58) mRNAs upon H19 silencing in VUMC-DIPG-A cells. (c) Process diagram for the identification of previously validated H19 miRNA targets with the lncRNA v5 database and a luciferase binding assay. (d) Trypan blue exclusion cell number on let-7a-5p-overexpressing VUMC-DIPG-A cells on days 3 and 5 post-transfection. (e) Brightfield images of the let-7a-5p mimic and the control (scramble oligonucleotide) overexpressing VUMC-DIPG-A cells (\( n = 4 \)). Magnification 10×. (f) Venn diagram of cross-referencing H19-silenced mRNAs with let-7a-5p-predicted mRNA targets. Overlapping mRNAs were analysed for clinical correlation with H19 in paediatric Cbioportal. (g) Correlation analysis of H19 expression and SULF2 or OSMR in the ICR 2017 pedHGG dataset on the paediatric Cbioportal platform [25]. (h) Graphical representation of predicted binding site between let-7a-5p and SULF2 (top) or OSMR (bottom) mRNA. (i) SULF2 and OSMR expression in H19-silenced VUMC-DIPG-A cells 2 days after transfection, analysed by qPCR (\( n = 3 \)). Data on (b) were analysed with a two-way ANOVA and Sidak’s post-hoc multiple comparison. Data on (i) were analysed with an unpaired two-tailed \( t \)-test. Data on (g) were analysed using Spearman’s correlation. Data are shown as mean ± SD and ** \( p < 0.01 \).
To identify which mRNAs are likely to be targeted by both H19 and let-7, the list of H19-down-regulated mRNAs (622) was cross-referenced with the list of let-7a-5p-predicted mRNAs (1207) extracted from TargetScan 7.0 (Figure 3f). There were 64 overlapping mRNAs, but only eight of these were positively and significantly (p < 0.0001) correlated with H19 expression in clinical samples using the data of pedHGGs from paediatric Cbioportal. SULF2 and OSMR were the top two protein-coding genes that correlated with H19 expression (Supplementary Table S4). Let-7a-5p had a predicted 6mer and 8mer binding site for SULF2 and OSMR, respectively (Figure 3g). In keeping with our mechanistic model, the expression of SULF2 (p < 0.01) and OSMR (p = 0.052) was reduced in H19-silenced VUMC-DIPG-A cells (Figure 3h). These results suggest that H19 potentially sponges let-7a-5p thereby, causing the up-regulation of let-7a-5p-target mRNAs (such as SULF2 and OSMR).

3. Discussion

In this study, we showed that H19 is up-regulated in DIPGs, and that silencing H19 decreases DIPG-H2K27M cell proliferation and increases caspase 3/7 activity. To the best of our knowledge, this is the first functional characterisation of a lncRNA in DIPGs. The oncogenic role of H19 has been previously proposed for a wide range of malignancies, including adult high-grade gliomas [15]. In keeping with our findings, several lines of evidence showed that H19 silencing leads to decreased cell proliferation in other malignancies; interestingly, this effect was explained by a wide range of mechanisms of action, which appear to be, at least in part, cell-specific [28–30].

Herein, we showed an association between H19 expression and DIPGs bearing H3K27M. The molecular landscape of DIPGs is very heterogeneous [4]. Besides H3K27M, mutations in other chromatin regulators have been identified in DIPGs, including mutations in the ATRX and DAXX genes. These mutations are not common, but have been suggested to be associated with DIPG-H3K27M; however, their implication in DIPG progression and their link with H3K27M are poorly understood [4,21]. Other chromatin changes are controlled by dynamic post-translational modifications (PTMs) on the histone N-terminal tail, which influence the structure of chromatin and, hence, gene expression [31]. DIPG tumours bearing H3K27M induce a dramatic reduction in the global levels of H3K27me3 in DIPG cells [4]. Recently, other PTMs were identified, including H3K26me2 and H416ac [31]. Herein, we did not have the bioinformatics tools to determine the association between histone PTMs and H19 expression, but this would be very interesting and the objective of future studies.

The origin of DIPGs is controversial, but recent research suggests that these malignancies originate from the partial differentiation of neural stem cells into oligodendrocyte precursor (OPC)-like cancer cells [4,32]. Herein, we showed that H19 is up-regulated in OPC cells during brain development. In keeping with this finding, it has been shown that H19 is down-regulated upon full differentiation of OPCs into oligodendrocytes [33]. Taken together, our results and previous publications suggest that H19 may be implicated in the proliferation of OPC-like cancer cells, which are the cells of origin in DIPGs.

Mechanistically, H19 is a cytoplasmic lncRNA that can sponge miRNAs to modulate gene expression [26]. Several miRNAs have been shown to directly interact with H19, including miR-141-3p, miR-200b-3p, and let-7a [34–36]. It is therefore likely that the effects of H19 on normal and cancer cells are determined by the complex interplay of a pool of cell-specific miRNAs expressed in each cell type. To discover the miRNAs that mediate H19 function in DIPGs, we crossed the list of H19 targets with the list of miRNAs able to bind to the mRNAs down-regulated upon H19 silencing. Based on this analysis, we decided to further investigate let-7a-5p, because this miRNA has been shown to interact with H19 in other contexts [36–38] and to down-regulate 64 H19-dependent mRNAs. In keeping with our hypothesis, overexpression of let-7a-5p led to a reduction in DIPG cell proliferation; this observation suggests that H19 buffers the onco-suppressive let-7a-5p, thereby increasing DIPG cell proliferation. However, let-7a-5p overexpression was not as
cytotoxic as that of \textit{H19} silencing; this observation suggests that \textit{let-7a-5p} is only partially responsible for \textit{H19} oncogenic function.

Interestingly, \textit{SULF2} increases cell proliferation, invasion, mobility, and adhesion in MCF-7 and MDA-MB-231 breast cancer cell lines [39]. \textit{OSMR} promotes proliferation, metastasis, and EMT in prostate cancer [40]. \textit{OSMR} also increases mitochondrial respiration and radio-resistance in glioblastoma stem cells [41].

Cell proliferation is defined as the balance between cell survival and cell death. Our results indicate that \textit{H19} is able to drive cell survival and suppress cell death in DIPG cells. However, \textit{let-7a-5p} seems only to affect cell growth. Therefore, it seems likely that other miRNAs mediate the anti-apoptotic function of \textit{H19} in DIPGs.

The primary aim of this work was to functionally characterise a DIPG-specific lncRNA, and to identify therapeutic targets for these incurable malignancies. For this reason, silencing experiments were carried out with LNAs instead of more conventional siRNAs. LNAs are more stable in biological media and are currently being investigated in clinical trials for the treatment of neurological disorders and malignancies [42–45]. Herein, we reported that an \textit{H19}-targeting LNA (LNA2) has an in vitro IC\(_{50}\) lower than the typical maximum plasma concentrations of LNAs in clinical trials (2000–6000 ng/mL) [46]. Future in vivo studies are warranted to establish whether LNA2 is effective in clinically relevant animal models of DIPG-H3K27M.

In conclusion, this work has shown, for the first time, the role of an oncogenic lncRNA in DIPGs, which is likely to control multiple pathways in DIPG-H3K27M. We hope this work will encourage DIPG researchers to further explore the function of lncRNAs and to test their role as therapeutic agents.

4. Materials and Methods
4.1. Bioinformatic Analysis
4.1.1. R2 Platform Analysis

\textit{H19} lncRNA was queried in the R2: Genomics Analysis and Visualization Platform (https://hgserver1.amc.nl/cgi-bin/r2/main.cgi, accessed on 31 December 2020) to assess its clinical relevance in DIPG tissue and normal brain tissue. The datasets used for this analysis were GSE26576 (R2 ID: ps_avgpres_gse26576geo37_u133p2, referred to as “Paugh”) and GSE50021 (R2 ID: ps_avgpres_gse50021geo45_imnht12v4, referred to as “Allis”) [23,24]. Data were sorted based on “disease_type” (Paugh) and “cell_type” (Allis). Only DIPG and normal brain tissues were compared.

4.1.2. Paediatric Cbioportal Analysis

The paediatric Cbioportal analysis was carried out with \textit{H19} lncRNA (https://pedcbioportal.kidsfirstdrc.org/, accessed on 31 December 2020) to assess the difference in the expression of \textit{H19} in paediatric high-grade gliomas. Data were obtained from the publicly available Institute of Cancer Research (ICR) dataset from 2017 [25] and sorted based on either (1) the type of histone modification (wild type (WT); H3.1K27M and H3.3K27M, grouped as H3K27M), (2) brain location (hemispheric, midline, or brainstem), or (3) histological classification (DIPGs or other paediatric high-grade gliomas, including anaplastic gliomas and glioblastomas). Data were also used to perform Kaplan–Meier analysis sorted based on the median expression of \textit{H19} in Graphpad Prism 7 (Graphpad Software, San Diego, CA, USA).

4.1.3. Single-Cell Analysis

Open access dataset on single-cell analysis of the midbrain in development (http://linnarssonlab.org/ventralmidbrain/, accessed on 31 March 2021). \textit{H19} was queried and its expression in human cells was analysed.
4.2. Cell Culture

Primary human foetal cortical normal astrocytes were a gift from Prof. DK Male (The Open University, Milton, Keynes, UK) and were cultured as described elsewhere [47]. The VUMC-DIPG-A (H3.3 K27M) cell line was kindly provided by Dr. Esther Hulleman (VUMC Cancer Center, Amsterdam, the Netherlands) [22]. SU-DIPG-IV (H3.1 K27M) cells were kindly provided by Dr. Michelle Monje (Stanford University, California, USA). [48]. VUMC-DIPG-A cells were cultured in 1:1 DMEM-F12 and Neurobasal-A cell media and supplemented with a 1% (v/v) glutamax supplement, a 1% (v/v) antibiotic-antimycotic solution, 10 mM HEPES, a 1% (v/v) MEM non-essential amino acid solution, and 1 mM sodium pyruvate (Thermofisher, Paisley, UK), hereafter referred to as TBM medium. VUMC-DIPG-A medium was also supplemented with 10% heat-inactivated foetal bovine serum (Merk, Watford, UK). SU-DIPG-IV was cultured in TBM medium supplemented with a 2% (v/v) B27 supplement without vitamin A (Thermofisher), 20 ng/mL of bFGF, 20 ng/mL of EGF, 10 ng/mL of PDGF-AA, 10 ng/mL of PDGF-BB (Peprotech), and 5 IE/mL of heparin (Merk). Cells were grown in adherence and passaged using 0.25% (v/v) trypsin-EDTA (Merk). All cells were cultured at 37 °C in a humidified environment containing 5% CO₂.

4.3. Analysis of Gene Expression

RNA was isolated from cells in the culture using the RNeasy Plus Mini Kit (Qiage, Manchester, UK) according to the manufacturer’s instructions. Reverse transcription was carried out using 1 µg of RNA per reaction using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Life Technologies, Warrington, UK) according to the manufacturer’s instructions. For RT-qPCR analysis, 10 ng of cDNA were loaded in duplicate per sample using the Taqman Universal mastermix and primers. The Taqman primers used in this study were H19 (Hs00399294_g1), GADPH (Hs02786624_g1), SULF2 (Hs01016480_m1), and OSMR (Hs00384276_m1).

4.4. Subcellular Localisation of H19

RNA was isolated from cells in the culture in nuclear and cytoplasmic fractions, which were separated using the PARIS Kit (Ambion, Thermofisher, Paisley, UK) according to the manufacturer’s instruction. For validation and localisation studies, RT-qPCR was used with the probes MALAT1 (Hs00273907_s1), GAPDH (Hs02786624_g1), and HPRT1 (Hs02800695_m1).

4.5. LNA Reverse Transfection

Knockdown studies were performed using the reverse transfection method. Cells were seeded in a 6-well (180,000 cells/well) or 96-well plate (5000 cells/well) with a lipid/LNA mixture prepared using Oligofectamine (Invitrogen, Thermofisher, Paisley, UK) as per the manufacturer’s protocol. The final LNA concentrations were 100 nM. All LNAs were designed using the Qiagen LNA design tool and the targeting sequences were ACTAAAT-GAATTGCGG (LNA 1), AATTCAGAAGGGACGG (LNA 2), and GACTTAGTGCAAATTA (LNA 3). Control LNA were also purchased from Qiagen.

4.6. Cell Proliferation Assay

LNA-transfected cells were seeded on 6-well plates and grown at specific times, as specified in the figure legends. On the day of analysis, cells were trypsinised, centrifuged, and resuspended in HBSS for cell counting using the trypan blue (Merk) exclusion method with either a cell haemocytometer or an automatic cell counter (LUNA, Logos Bioscience, Villeneuve-d’Asq, France).

4.7. Caspase 3/7 Assays

Cells were plated in a white, flat-bottomed 96-well plates and treated with 100 nM of LNAs, as previously described. On day 4 post-transfection, the Caspase-Glo reagent was added to each well (Promega, Southampton, UK) according to the manufacturer’s
instruction and incubated for 1.5 h. Luminescence was then quantified using the BMG polarStar plate reader (BMG Labtech, Aylesbury, UK).

4.8. Wound Healing Assay (Migration)

VUMC-DIPG-A cells were reverse transfected for 18 h with 50 nM LNA2, as these did not yield a significant reduction in viable cells as determined by cell proliferation assays. After 18 h, a scratch was made in the cell monolayer using a sterile P20 pipette tip. Cells were imaged periodically across the wound until the wound was closed. Images were analysed using the ImageJ (ImageJ Software, Madison, WI, USA).

4.9. RNA Sequencing Analysis

VUMC-DIPG-A cells were reverse transfected with 100 nM of LNA and RNA isolated 48 h post-transfection. NGS libraries were prepared according to the Ion AmpliSeq Library Kit Plus’ user guide (Thermo Fisher Scientific, Paisley, UK). For each sample, 100 ng of total RNA was reverse transcribed using the SuperScript VILO cDNA Synthesis Kit (Thermo Fisher Scientific). The resulting cDNA was then amplified for 10 cycles by adding PCR Master Mix and the AmpliSeq Human Transcriptome Gene Expression Kit panel, targeting over 20,000 genes (>95% of the RefSeq gene database). Amplicons were digested with the proprietary FuPa enzyme to generate compatible ends for barcoded adapters ligation. The resulting libraries were purified using AmpureXP beads (Agencourt, Beckman Coulter Life Sciences, Nyon, Switzerland) at a bead-to-sample ratio of 1.5× and eluted in 50 µL of low TE buffer. The libraries were then diluted 1:10,000 and quantified by qPCR using the Ion Universal Quantitation Kit (Thermo Fisher Scientific). Individual libraries were diluted to a 50 pM concentration, combined in batches of eight libraries, loaded on an Ion 540™ chip using the Ion ChefTM instrument and sequenced on an Ion SS™ instrument (Thermo Fisher Scientific). QC was manually performed for each sample based on the following metrics: Number of reads per sample >8 × 10⁶, with valid reads >90%. Raw data were processed automatically on the Torrent Server™ and aligned to the reference hg19 AmpliSeq Transcriptome fasta reference.

The AmpliSeqRNA plugin was used to determine valid matches to the amplicon target regions in the panel using the hg19 AmpliSeq Transcriptome bed file. Each sample had more than 5 × 10⁶ total aligned reads. At least 10 mapped reads per amplicon in more than three samples were used to filter out lowly covered amplicons. Two analysis pipelines were used separately. Log-fold changes reported by DESeq2 (10.1186/s13059-014-0550-8) were shrunk with apeglm (10.1093/bioinformatics/bty895), available within the DESeq2 Bioconductor package (package version: 1.30.1, 10.18129/B9.bioc.DESeq2). Genes were reported as significant with log-fold changes >0.58 and s-values <0.01. Analyses were performed in R (version: 4.0.3, R Core Team (2020) using RStudio interface (version 1.3.1073)).

Biological function gene ontology was analysed with Gene Set Enrichment Analysis (http://www.gsea-msigdb.org/gsea/msigdb/index.jsp, accessed on 30 April 2021) and the “Hallmark” feature using genes grouped as up-regulated (log2FCs >0.58 and s-values <0.01) or down-regulated (log2FCs <−0.58 and s-values <0.01).

4.10. miRNA Mimic Studies

VUMC-DIPG-A cells were reverse transfected with 30 nM negative control (NC; scramble control) or the let-7a-5p mimic using the method previously described. The sequences used were obtained from Thermo Fisher and were as follows: let-7a-5p mimic (MC31809) and NC mimic (4464058).

4.11. Analysis of miRNA Expression

For miRNA analysis, RNA was isolated using miRNeasy (Qiagen) according to the manufacturer’s instructions. Reverse transcription was carried out using the miRNA cDNA Synthesis Kit (Applied biosystems, Paisley, UK) according to the manufacturer’s
instructions using the reverse transcription primers provided with let-7a-5p (000377) or U6 snRNA (001973) using 10 ng of RNA per reaction. Then, 1.7 ng of the resulting cDNA was used for the qPCR analysis using the qPCR taqman probes provided in the kits described for let-7a-5p or U6 according to the manufacturer’s instructions.

4.12. In Silico Prediction of miRNA Target Genes

Identification of the experimentally validated miRNA–lncRNA binding site was carried out with the online open access lncBase v.3 database (http://carolina.imis.athenainnovation.gr/diana_tools/web/index.php?r=site%2Ftools, accessed on 30 September 2020) [49]. Only miRNAs whose binding has been previously demonstrated by luciferase assay were taken into account. Prediction of miRNA–mRNA interactions was investigated using the open access TargetScan database (http://www.targetscan.org/vert_72/, accessed on 30 September 2020). The predicted target mRNAs were overlapped with mRNAs down-regulated upon H19 knockdown (RNA-seq) in VUMC-DIPG-A cells. Top mRNAs that were positively correlated (Spearman values <0.05) with H19 expression in the pedHGG clinical dataset (paediatric Cbioportal, ICR 2017 pedHGG study) were prioritised for validation.

4.13. Statistical Analysis

All data are presented as means ± SDs (standard deviations). The number of independent experiments (n) with replicates are specified in each legend. Normality was assessed with Shapiro–Wilk test (p = 0.05). Means were compared using unpaired or paired two-tailed t-tests for single comparison and one-way or two-way ANOVAs for multiple comparisons. ANOVA analysis was followed by post-hoc analysis. Specific post-hoc analysis is specified in each figure legend. All tests were performed using the statistical software GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA). A p-value <0.05 was considered to be statistically significant.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ijms22179165/s1.


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