A microRNA profile of pediatric glioblastoma: The role of NUCKS1 upregulation

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Abstract. MicroRNAs (miRNAs/miRs) are a novel class of gene regulators that may be involved in tumor chemoresistance. Recently, specific miRNA expression profiles have been identified in adult glioblastoma (aGBM), but there are only limited data available on the role of miRNAs in pediatric GBM (pGBM). In the present study, the expression profile of miRNAs was examined in seven pGBMs and three human GBM cell lines (U87MG, A172 and T98G), compared with a non-tumoral pool of pediatric cerebral cortex samples by microarray analysis. A set of differentially expressed miRNAs was identified, including miR-490, miR-876-3p, miR-876-5p, miR-448 and miR-137 (downregulated), as well as miR-501-3p (upregulated). Through bioinformatics analysis, a series of target genes was predicted. In addition, similar gene expression patterns in pGBMs and cell lines was confirmed. Of note, drug resistant T98G cells had upregulated nuclear casin kinase and cyclin-dependent kinase substrate 1 (NUCKS1) expression, a protein overexpressed in many tumors that serves an important role in cell proliferation and progression. On the basis of the present preliminary report, it could be intriguing to further investigate the relationship between each of the identified differentially expressed miRNAs and NUCKS1, in order to clarify their involvement in the multi-drug resistance mechanism of pGBMs.

Introduction

Glioblastoma (GBM) is a highly aggressive, invasive and poorly responsive brain tumor. At present, the median survival time for children that have received chemotherapy and radiotherapy is reported between 11-24 months; the five-year survival rate is below 20% (1). Adult (aGBM) and primary pediatric GBM (pGBM) show distinct molecular pathways of tumorigenesis and genetic profiling (1-6); pGBM appears to be more similar to secondary aGBM that has evolved from diffuse grade II or grade III gliomas. Indeed, similarly to secondary aGBM, pGBM exhibits transcriptional regulator ATRX, histone H3.3 and cellular tumor antigen p53 mutations and only rarely shows epidermal growth factor receptor amplification/overexpression (7,8) or phosphatase and tensin homolog mutations (9).

Chemosensitivity is the main obstacle to successful chemotherapy for brain tumors. Drug resistance is the main cause of tumor recurrence and patient relapse; this phenomenon is associated with several biological mechanisms, including apoptosis, DNA damage and repair, epigenetic regulation, alteration in ATP-binding cassette transporter family and dysregulation of microRNAs (miRNAs/miRs). A limited number of studies have investigated the underlying mechanisms of pGBM chemoresistance, although in recent decades, increasing amounts of data report the involvement of miRNAs in drug sensitivity and chemoresistance (10-17).

miRNAs represent a novel class of gene regulators that are involved in several physiological processes, including cell differentiation, proliferation, stress response and anti-viral defense, as well as pathological conditions such as cancer (18). They are a large family of evolutionary conserved, short (19-24 nucleotides), single-stranded, non-coding RNAs that also exhibit strong tissue and cellular specificity to developmental stages. They are able to regulate gene expression at the post-transcriptional level, leading to mRNA degradation, with consequential downregulation of encoded protein by translational repression. miRNAs regulate 3% of the human genome and up to 30% of protein-coding genes (19,20). Many miRNAs could regulate multiple miRNAs and a single miRNA could regulate multiple mRNA targets (21).
The etiological role of miRNAs in tumor development is supported by the observation that half of miRNA genes are localized in cancer-associated genes, fragile genome sites (22) or regions that are often amplified (23), acting as tumor suppressors or as oncogenes, depending on which genes/pathways they control (24). Aberrant microRNA expression profiles have been identified in aGBM (25–32), but few studies have investigated the role of miRNAs in pGBM (33,34).

In order to better understand chemoresistance mechanism and regulation in high-grade glioma (HGG), the present study generated a microRNA profile of pGBMs, through a TaqMan® Human MicroRNA Array v2.0 approach. A set of differentially expressed miRNAs in pGBMs and in GBM cell lines (A172, U87MG and resistant-T98G) was identified, in comparison to non-tumor pediatric cerebral cortex samples. The present preliminary study may contribute the biological understanding of pGBM chemoresistance, which represents the most common causes of relapse (35), and may provide biomarkers for therapeutic strategies.

Materials and methods

Patients and samples. Patients with pGBM seen between April 2008 and May 2013 at the Meyer Children's University Hospital (Florence, Italy) were eligible for the present study. Histological diagnosis and tumor grading was performed based on the 2007 World Health Organization criteria (36). In total, five non-tumor pediatric cerebral cortex samples (non-tumoral pool) and seven pGBMs were obtained at the Neuro-Surgery Unit of the Meyer Children's University Hospital. The present study was approved by the institutional Ethical Committee. Informed consent was obtained from the parents or legal guardians in all cases. Diagnosis was confirmed by the review of the CNS national panel of pathologists (Umberto I, Policlinico General Hospital Sapienza University, Rome, Italy). The median age at the time of diagnosis was 8±4.6 years (age range, 1-15 years; 4 females and 3 males). All patients had been treated with chemotherapy and/or radiotherapy according to consolidated pediatric treatments (37-39) and underwent surgery for resection of disease. The median follow-up was 10±6.1 months (range, 3-24 months).

Cell lines. Three human GBM cell lines, A172 (CRL-1620™; ATTC, Manassas, VA, USA), U87MG (HTB-14™; ATTC) and resistant-T98G (CRL-1690™; ATCC) were obtained. U87MG (HTB-14™; ATCC) and resistant-T98G (CRL-1690™; ATCC) were obtained at the Neuro-Surgery Unit of the Meyer Children's University Hospital. The present study was approved by the institutional Ethical Committee. Informed consent was obtained from the parents or legal guardians in all cases. Diagnosis was confirmed by the review of the CNS national panel of pathologists (Umberto I, Policlinico General Hospital Sapienza University, Rome, Italy). The median age at the time of diagnosis was 8±4.6 years (age range, 1-15 years; 4 females and 3 males). All patients had been treated with chemotherapy and/or radiotherapy according to consolidated pediatric treatments (37-39) and underwent surgery for resection of disease. The median follow-up was 10±6.1 months (range, 3-24 months).

Expression study. miRNA and mRNA were extracted using the mirVana™ miRNA Isolation kit (cat. no. AM1560; Thermo Fisher Scientific, Inc.) from tumor (pGBM 1-7) and non-tumor pediatric cerebral cortex samples (pool of 5 samples), as well as pellets of the three cell lines (A172, U87MG and resistant-T98G). Cells were trypsinized (Trypsin-EDTA 1X, cat. no. EGB3052D, Euroclone SpA) from the culture surface (6-well Primo multiwell plate; Euroclone SpA) and transferred to 15 ml conical tubes (TC Tube, 15 ml; SARSTEDT AG & Co. KG, Nümbrecht, Germany). The tubes containing cells and media were centrifuged at 800 x g for 5 min at 4°C to pellet cells and decant culture media. Subsequently, cells were washed in PBS (cat. no. ECB4004L; EuroClone SpA) and further centrifuged at 800 x g for 5 min at 4°C for pelleting. Finally, PBS was decanted and cell pellets were stored at -80°C.

MicroRNA expression profiles of three pGBMs and non-tumor pool of 5 samples were generated using TaqMan® Human MicroRNA A Cards v2.0 (cat. no. 4398977; Thermo Fisher Scientific, Inc.) according to manufacturers' protocol, using the 7900HT Fast Real-Time PCR system (Thermo Fisher Scientific, Inc.).

Bioinformatic analysis. Raw data were analyzed with the R computational environment by using the HTqPCR package version 1.0 (40). The package HTqPCR is designed for the analysis of cycle threshold (Ct) values from quantitative PCR (qPCR) data. The heatmap was generated using the R version 3.5.1 (41) and the HTqPCR package. Raw data were first normalized by using the quantile normalization approach and then analyzed for differential expression with the two-tailed t-test. miRNAs with statistically significant differential expression were analyzed with the miRanda algorithm (www.microrna.org; version 3.3a) to search for miRNA gene targets (42).

Finally, gene targets were analyzed for enrichment in Gene Ontology (www.geneontology.org) (43,44) and Kyoto Encyclopedia of Genes and Genomes database (www.genome.jp/kegg/kegg1.html) (45-47) with a Fisher's exact test.

Validation of miRNAs by quantitative polymerase chain reaction (qPCR). miRNA was extracted using the mirVana™ miRNA Isolation kit (cat. no. AM1560; Thermo Fisher Scientific, Inc.) from tumors (pGBM 1-7) and non-tumor pediatric cerebral cortex samples (pool of 5 samples), as well as pellets from three cell lines (A172, U87MG and resistant-T98G). The expression of previously identified dysregulated miRNAs were determined using commercial assays (miR-137, cat. no. 001129; miR-216a, cat. no. 477976; miR-490, cat. no. 001037; miR-501-3p, cat. no. 002435; miR-521, cat. no. Hs99999903_m1; miR-525-3p, cat. no. 478995_mir; miR-873, cat. no. 478204_mir; miR-876-3p, cat. no. 002225; miR-448, cat. no. 001029; all, Thermo Fisher Scientific, Inc.; included forward and reverse primers).

cDNA was synthesized using the TaqMan® MicroRNA Reverse Transcription kit (cat. no. 4366596; Thermo Fisher Scientific, Inc.) according to the manufacturers' protocol (30 min at 16°C, 30 min at 42°C and 5 min at 85°C) with the GeneAmp® PCR System 9700-Applied Biosystems (Thermo Fisher Scientific, Inc.).

qPCR was performed using the aforementioned commercial, ready to use assays according to the protocol instructions (10 min at 95°C, 40 cycles at 15 sec at 95°C, 60 sec at 60°C).
of the 7900HT Fast Real-Time PCR system (Thermo Fisher Scientific, Inc.). All assays were performed in triplicate. For each miRNA, the expression was normalized to that of RNU48 (cat. no. 001006; Thermo Fisher Scientific, Inc.) and calculated using the 2^\(-\Delta\Delta C_q\) method (48). GBMs were subsequently normalized and compared with the non-tumoral pool.

Validation of target genes by SYBR-Green PCR. mRNAs were extracted using the mirVana™ miRNA Isolation kit (cat. no. AM1560; Thermo Fisher Scientific, Inc.) from tumor (pGBM 1-7) and non-tumor pediatric cerebral cortex (pool of 5 samples) samples, as well as pellets (obtained as aforementioned) of the three cell lines (A172, U87MG and resistant-T98G). cDNA was synthesized using the High Capacity RNA-to-Cdna kit (cat. no. 4387406; Thermo Fisher Scientific, Inc.) according to the manufacturers' protocol (60 min at 37°C and 5 min at 95°C) with the GeneAmp® PCR System 9700-Applied Biosystems (Thermo Fisher Scientific, Inc.). Primers were designed using Primer3web version 4.1.0; (http://primer3.ut.ee/). The primer sequences utilized are presented in Table I. Validation of the expression of hypothetical target genes was performed using the LightCycler® 480 SYBR-Green I Master mix (Roche Diagnostics, Basel, Switzerland) on a LightCycler® 480 II (Roche Diagnostics) according to the manufacturers' protocol (10 min at 95°C, 40 cycles at 15 sec at 95°C, 45 sec at 60°C, 60 sec at 72°C; melting curve at 10 min at 95°C, 60 sec at 65°C) and quantification was obtained using 2^\(-\Delta\Delta C_q\) method (48).

Statistical analysis. Statistical analysis of nuclear casein kinase and cyclin dependent kinase substrate 1 (NUCKS1) expression was performed using one-way analysis of variance followed

| Primer   | Forward sequence (5'‑3') | Reverse sequence (5'‑3') |
|----------|-------------------------|-------------------------|
| GRIA1‑EXON13 | AGTCAGCAGAGGCTACAGTT | TGGGTGTGGCAATGCTGATAG |
| GRIA1‑EXON10 | CGTTACGAGGGCTACATGTG | TCCCTAGACAGGCTGTCCTCC |
| SORL1‑EXON46 | TCACAGCTACCTTGGGAACTC | GACCCCGACTCATCTGACAG |
| SORL1‑EXON35 | TGTCAGGAGAGGCAATGGGA | GTTCTCACTGACCCACTCC |
| NUCKSI‑EXON5 | AAAATGGTGCCCAAAACACC | ATATGTCGCTTACACTCTC |
| NUCKSI‑EXON7 | GTCACAAGGAGAGCAAGG | TCAAGACCTTCTATCCCAAG |
| SOX11‑EXON1 | ATATTTCCTCAGGAGCAGCA | GTGCACTAGTGGGAGACTC |
| SOX11‑EXON1.2 | ACATCAAGGGCCAGGGAG | GATGACAGGCTGAGGGTTC |
| SAP30L‑EXON1 | GCCTGACGAGGAGGAGGA | CTTCCTGGAGAGAGGATAG |
| SAP30L‑EXON4 | GCAGCCTCAAGGAGATCTTC | CACCCTGGATCTGAGTCC |
| HTT‑EXON63 | TGTTGGGAGAGAGCATATGGA | GGAGGTGGATCTGAGTCC |
| HTT‑EXON48 | GTTCAACCTACGGCTGACAG | GGTGACAGGCTGAGGGTTC |
| PXMP4‑EXON3 | TCAGGGCCATATATGCTACT | TGCTGACAGGCTGAGGGTT |
| PXMP4‑EXON4 | CGTTAGCTGAGAAGGAGGCTA | TACGGGCTTGGGACAGTT |
| THRB‑EXON8 | GAGAGAACAGGGCCAGGGGAAGA | GGGGGCTTGGGACAGGGA |
| THRB‑EXON10 | GCCTGATACCCCAAGAAATG | GATGATAGAGGCTGAGGG |
| PSD3‑EXON5 | TCTGAATGGGAGAGGACCTA | TCGTGGCTGACGGCTGAT |
| PSD3‑EXON11 | ACTGAGGAGAAGATGCTAGG | TCCCTACATCATCTGAGTC |
| SPN‑EXON2 | CTTAAGCTTCTTCTGACATT | CTGTCAGTGGGAGGAGG |
| SPN‑EXON2.1F | GTGACAGTGGACCTGTTGGGAG | GACCCAGACTTCTGCTTCC |
| AGPAT4‑EXON2 | ACGTGGGTTCTATGCTGAGTC | AGAGCAGTCTGCTGAGTTC |
| AGPAT4‑EXON4 | AAGCTTCTGGCCAAAGAAAGA | AAAATCTATCTCGGAGTTGG |
| USP31‑EXON11 | AGACGAGGCGCATGAAACTT | ATGATGCTTACGGGTTCCT |
| GRK3‑EXON3 | CAAATGGGTCCTGAGCTTC | CTGCTGACAGGCTGAGGGT |
| GRK3‑EXON14 | TTCGAGAGATGTGGGCTCCT | AATGAGGGATGCTGAGTTC |
| TNRC6B‑EXON11 | CAAATCTACAGTGGGCTCTT | CTGAAGCTGCTGAGTTTGG |
| TNRC6B‑EXON20 | TGTCCTCTACAGTCATCGG | GATCCTGTCGCTGAGTTC |
| SNX29‑EXON5 | CCGTTGTTGCTGATCTACCTT | GGAGTGAGTGTGGGAGG |
| SNX29‑EXON8 | CAAATGGGAGGAGGAGGAGC | CCTGTCCTCCTCCTCCT |
| HIPK2‑EXON2 | CGTGCTTGGTCTGAGATGC | GCTGCTGCTGAGTGGCCT |
| HIPK2‑EXON13 | CCCTAGCTCCGACTCTTC | ACCAAATCTCGTCTTC |
| RIMKLA‑EXON1 | CAGTCTCTGCTCCTGAGC | GGTGATCTTCGATAGC |
| RIMKLA‑EXON5 | TGACAGACAGGAGCAG | GCAATGACCGCACTACATC |
Results

Bioinformatics analysis. Bioinformatics analysis of TaqMan® Human MicroRNA array version 2.0 revealed a set of miRNAs (miR-137, miR-216a, miR-490, miR-501-3p, miR-521, miR-525-3p, miR-672, miR-873, miR-876-3p and miR-448) that exhibited a statistically significant differential expression in three pGBMs (GBM 1-3) when compared with the non-tumoral pool (Fig. 1; significance cut-off level, two-tailed t-test with P<0.01).

Validation of miRNAs and target genes. In all tumors (pGBMs 1-7) and GBM cell lines (U87MG, A172 and resistant-T98G), the downregulation of miR-137, miR-490, miR-876-3p and miR-448 was confirmed, and the upregulation of miR-501-3p was demonstrated.

Concerning the expression of the other dysregulated miRNAs (miR-216a, -521, -525-3p, -672 and -873), the interpretation of these results was unsuccessful due to poor reaction efficiency of the commercial assays.

Discussion

In the present study, a microRNA expression profile of pGBM was generated using TaqMan® Human MicroRNA Array v2.0. in GBM tumors (7 pGBMs) and cell lines (U87MG, A172, resistant-T98G). The results demonstrated that miR-137, miR-490, miR-876-3p, miR-876-5p and miR-448 were downregulated, and miR-501-3p was upregulated. Concerning the expression of the other dysregulated miRNAs, the interpretation of these results was unsuccessful due to the poor reaction efficiency of the commercial assays used.

Furthermore, it was determined that the aforementioned miRNAs were involved in the regulation of the following target genes: GRIA1, SORL1, NUCKSI, SOX11, SAP30L, HTT, PXMP4, THR, PSD3, SPN, AGPAT4, USP3, GRIK3, POM121L8P, TNRC6B, SNX29, HIPK2 and RIMKLA. All
hypothetical target genes were identified in tumors and cell lines and the overexpression of NUCKSI was detected in drug resistant T98G cells.

NUCKSI is a highly phosphorylated nuclear DNA-binding protein that is involved in cell cycle progression and proliferation (49). It serves as a substrate for casein kinase 2 and cyclin-dependent kinase (CDK) -1, -2, -4 and -6 (49-53). NUCKSI also serves a role in the response to DNA damage, homologous recombination and DNA repair mechanisms that are critical for tumor suppression (54). The increased expression of NUCKSI has been reported in several different types of cancer, including breast, colorectal, cervical and hepatocellular carcinoma (50,55-57). However, its exact role in cancer development remains unclear. The NUCKSI gene is located on chromosome 1q32.1 (chr 1, 205,712,819-205,750,276), which undergoes recurrent duplication/amplification in several different types of tumor (58,59), including that of the brain (60-63). It is well established that genes amplified in specific copy number variants are associated with tumor progression and poor prognoses (58-63).

Recently, Shen et al (64) demonstrated that NUCKSI was a target of miR-137 in human lung cancer tissue and resistant lung cell lines. They also revealed that the tumor suppressive role of miR-137 is mediated via the negative regulation of NUCKSI protein expression. miR‑137 is a tumor suppressor and a number of its target genes, including cell division control protein 42, CDK6, cyclooxygenase-2, paxillin, AKT2 and induced myeloid leukemia cell differentiation protein are involved in cancer pathogenesis (64). The loss of miR‑137 expression has been determined in several different types of tumor (65-69), including GBMs (28,30,32,69-72). In addition, the restoration of miR‑137 expression has been demonstrated to be associated with the inhibition of tumorigenesis (64). In glioma cell lines that overexpress miR‑137, cell cycle arrest in the G1 phase is promoted via CDK6 suppression and retinoblastoma-associated protein-1 phosphorylation (26).

miR‑137 expression increases during the glioma stem-like cell differentiation in neurosphere cultures (70). The low expression of miR‑137 observed in GBM may reflect the loss of tumor cell differentiation, which may contribute to an increased cell proliferation, whilst maintaining an undifferentiated state (70).

At present, few data assess the differential expression of the remaining miRNAs that were determined in the present study. miR-490 is involved in the development and invasion of different types of tumor (73-76) and in the drug resistance of ovarian cancer (77). miR-448 functions as a tumor suppressor gene in osteosarcoma, where it is downregulated in tissues and in vitro models (78). miR-448 is also downregulated in hepatocarcinoma and is associated with tumorigenesis (79). This association has also been reported in ovarian cancer tissues and cell lines (80), breast cancer (81) and in T-cell acute lymphoblastic leukemia (82). Conversely, miR-488 is overexpressed in lung cancer (83).

miR-501-3p has been determined to be a potential biomarker associated with the progression of Alzheimer's disease (84). In cancer however, it may serve as a potential biomarker for pancreatic ductal adenocarcinoma (85) and lymph node metastasis in gastric cancer (86). Additionally, miR-876-3p/5p has been associated with papillary thyroid carcinoma (87), Hodgkin's lymphoma (88) and lung cancer (89). The miR-876 gene is located on the 9p chromosome, which is deleted in various types of cancer (90,91). Furthermore, pGBMs in particular exhibit a recurrent homozygous or heterozygous 9p21.3 deletion, including the MIR876 gene (60). Thus, the association between each of the dysregulated miRNAs, NUCKSI overexpression and chemoresistance mechanisms in pGBMs requires further investigation. Furthermore, it may be important to evaluate the role of NUCKSI protein expression in pGBM tumor progression in a larger and independent group of pediatric high grade glioma samples. NUCKSI overexpression in the resistant-T98G cell line, in comparison with other non-resistant cell lines, U87MG and A172, indicate its potential involvement in drug sensitivity and pGBM response. The T98G cell line represents a useful in vitro model, which may be utilized to determine the mechanism of acquired chemoresistance in patients with pGBM. In a previous study, it was demonstrated that T98G cells exhibit a different biological response to antineoplastic treatments (doxorubicin) compared with other GBM cell lines (92).

Chemoresistance represents an important challenge in pGBM treatment and overcoming this phenomenon may improve patient prognosis and increase survival rate. miRNAs are promising clinical biomarkers, which may produce a greater understanding of the biological processes associated with the development and progression of pGBMs.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LaG designed the current study and analyzed the data. LaG, BM, SL, VDG and MDR performed machine learning analysis. AM interpreted the data. LaG revised the work critically for important intellectual content. LaG and VDG made substantial contributions to drafting the manuscript. AMB histologically diagnosed the tumors. IS, LoG and SG supervised the study and were involved in patient recruitment, selection and treatment.

Ethics approval and consent to participate

The present study was approved by the institutional Ethical Committee of Meyer Children's Hospital, Florence, Italy. Informed consent was obtained from the parents or legal guardians in all cases.

Patient consent for publication

Written informed consent was obtained all the patients.
Competing interests
The authors declare that they have no competing interests.

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