Non-coding RNA are differentially expressed in diffuse intrinsic pontine gliomas based on overall survival

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Diffuse intrinsic pontine glioma is a pediatric brain cancer; 99% of patients diagnosed with this disease will expire within 5 years (1) and there are no standard chemotherapies available for it (2). Understanding the transcriptional behavior of this cancer is critical for the development of novel therapies. We performed global differential gene expression profiling of tumors from patients with DIPG by comparing the transcriptomes of tumors from those who survived greater or less than six months using a published dataset (3). We found that multiple microRNAs and snoRNAs were among the genes whose expression was most different between those who survived greater or less than six months. This is the first report of outcome-associated differential expression of non-coding RNA in diffuse intrinsic pontine glioma.

Keywords: diffuse intrinsic pontine glioma, DIPG, non-coding RNA, ncRNA, small nucleolar RNA, snoRNA, microRNA, miRNA, systems oncology, tumor transcriptome analysis, pediatric brain cancer.

Introduction

Diffuse intrinsic pontine glioma is a pediatric brain cancer that arises in the brainstem from the glial cells of the brain (1, 4, 5). Patients diagnosed with DIPG carry the worst of all cancer diagnoses - 99% will expire within five years and 100% of patients will expire within ten years (1). There is no standard chemotherapy that is considered acceptable (2), even for palliative use, and the only treatment regimen considered standard is radiation therapy (6). Understanding the basic biology of the cancer is critical to be able to identify therapeutic targets and for the development of novel therapeutic regimens. Towards this end, we performed here a global, differential gene expression analysis of tumors from patients with DIPG using a published dataset. By comparing the transcriptomes of patients that survived greater or less than six months, we found that multiple non-coding RNA transcripts were among the top 1% of transcripts that were most differentially expressed between these two groups of patients with differing overall survival outcomes. These included microRNA and small nucleolar RNAs, or snoRNAs. These results are the first to document differential expression of non-coding RNA in DIPG based on patient outcomes.

Methods

Dataset GSE50021 (3) was utilized for this analysis, in conjunction with GEO2R, to perform differential gene expression analysis on tumor samples from patients diagnosed with DIPG (n=35; n=21 for patients surviving greater than 6 months, and n=14 for patients surviving less than six months). There was one patient in this dataset who survived 6 months exactly and was included in the group of patients who survived less than 6 months. Buczkowicz et al. (3) used Illumina HT-12 microarray analysis to obtain transcriptome data from these tumors and control brain samples. These samples were all fresh frozen paraffin embedded tumors from pediatric patients, or normal healthy brain tissue. The p-value was not adjusted by any method. Log transformation was set to "No" and the submitter supplied category of platform annotation was used. For statistical analysis to compare the RNA expression values between

patient groups, an unpaired, two-tailed *t*-test with Welch's correction was used (PRISM 8.1.2) (227). Linear regression analysis and a Pearson correlation, comparing the mRNA expression level of each patient and the time amount of time they survived, were also performed using PRISM.

Results

We performed differential gene expression analysis to identify the genes whose expression changed the most between the tumors transcriptomes of patients who survived greater than and less than six months. Interestingly, ten separate non-coding RNA transcripts were among the top 1% of transcripts that were differentially expressed. These included five snoRNAs and five microRNAs.

Ten of the top 1% of differentially expressed genes when comparing the transcriptomes of those who survive greater or less than six months are non-coding RNA including snoRNA and microRNA.

There were five separate small nucleolar RNAs, or snoRNAs that were among the top 1% of transcripts differentially expressed in the tumor transcriptomes of patients surviving greater or less than 6 months. SNORD94 was the 14th most differentially expressed gene out of 29285 total transcripts detected and measured in the microarray data analyzed in this study (Table 1; $p=0.005574$) (3). SNORD100 was the 30th most differentially expressed gene out of 29285 total transcripts detected and measured (Table 1; $p=0.007554$). SNORD65 was the 65th most differentially expressed gene out of 29285 total transcripts detected and measured (Table 1; $p=0.0065$). SNORD49A was the 200th most differentially expressed gene out of 29285 total transcripts detected and measured (Table 1; $p=0.023731$).

There were five microRNA among the top 1% of transcripts differentially expressed between the transcriptomes of tumors from patients surviving greater or less than 6 months. miR939 was the 15th most differentially expressed gene out of 29285 total transcripts detected and measured (Table 1; $p=0.005638$). miR429 was the 29th most differentially expressed gene out of 29285 total transcripts detected and measured (Table 1; $p=0.007442$). miR595 was the

69th most differentially expressed gene out of 29285 total transcripts detected and measured (Table 1; $p=0.012938$). miR154 was the 140th most differentially expressed gene out of 29285 total transcripts detected and measured in the microarray data analyzed in this study (Table 1; $p=0.019527$). miR485 was the 197th most differentially expressed gene out of 29285 total transcripts detected and measured (Table 1; $p=0.023651$).

Next, we compared the expression levels of each non-coding RNA transcript in the tumors of all 35 patients, between the two groups, surviving greater or less than 6 months.

miR939 was expressed at significantly lower levels in the tumors of patients surviving greater than six months (Figure 1; $p=0.0222$). miR429 was expressed at significantly lower levels in the tumors of patients surviving greater than six months (Figure 2; $p=0.0338$). miR595 was expressed at significantly lower levels in the tumors of patients surviving greater than six months (Figure 3; $p=0.0351$). miR154 was expressed at significantly lower levels in the tumors of patients surviving greater than six months (Figure 4; $p=0.0436$). miR485 was expressed at significantly lower levels in the tumors of patients surviving greater than six months (Figure 5; $p=0.0691$).

In sum, each of five microRNA transcripts were expressed at significantly lower levels in the tumors of patients surviving greater than 6 months.

SNORA94 was expressed at significantly lower levels in the tumors of patients surviving greater than six months (Figure 6; $p=0.0341$). SNORD65 was expressed at significantly lower levels in the tumors of patients surviving greater than six months (Figure 7; $p=0.0250$). SNORD100 was expressed at significantly lower levels in the tumors of patients surviving greater than six months (Figure 8; $p=0.0320$). SNORD49A was expressed at lower levels in the tumors of patients surviving greater than six months (Figure 9; $p=0.762$). SNORA22 was expressed at significantly higher levels in the tumors of patients surviving greater than six months (Figure 10; $p=0.0046$).

In sum, all but one snoRNA, SNORA22, was expressed at lower levels in the tumors of patients surviving greater than six months.

Finally, we assessed whether the expression of any of these non-coding RNA transcripts displayed a linear correlation with the overall survival of the patient, measured by length of time the patient survived. The only ncRNA that whose expression displayed a linear and non-zero correlation with length of patient survival, and which approached the level of statistical significance to 95% confidence was SNORA22 (Figure 12E; $p=0.0822$). Surprisingly, this was also the only ncRNA, out of ten ncRNA transcripts, whose expression was higher in the tumors of patients surviving greater than six months.

Discussion

In this study, we performed global differential gene expression profiling to more thoroughly understand the transcriptional behavior of brainstem gliomas in diffuse intrinsic pontine glioma. By comparing the transcriptomes of tumors from patients that survived greater or less than 6 months, we found that 10 of the top 1% of transcripts that were most differentially expressed between the tumors of patients who survived greater or less than six months were non-coding RNA transcripts, both microRNA and snoRNAs.

These ncRNA transcripts can be used a diagnostic. More specifically, the levels of SNORA22 were significantly higher in the tumors of patients that survived over 6 months, and SNORA22 levels in each patients tumor also displayed a linear correlation with the amount of the time each patient survived, and this correlation, though not absolutely statistically significant to 95% confidence, approached statistical significance. SNORA22 belongs to the H/ACA box of snoRNAs whose function is to guide modification of uridine to pseudouridine in of substrate RNAs, specifically ribosomal RNAs (rRNAs) (7). SNORA22, as an exosomal RNA, was shown to be over expressed in pancreatic ductal adenocarcinoma (PDAC) (8). SNORA22 was also found to be differentially expressed between metastatic and non-metastatic xenografts in an analysis of prostate cancer (9).

miR939 has been implicated in the pathogenesis of breast cancer (10), ovarian cancer (11), gastric cancer (12) and hepatocellular carcinoma (13). miR154 has been implicated in prostate cancer (14, 15), colorectal cancer (16), and hepatocellular carcinoma (17), as well as

non-small cell lung carcinoma (18). miR429 has been implicated in the pathogenesis of non-small cell lung cancer (19), gastric carcinoma (20), ovarian cancer (21), colorectal cancer (22-23), bladder cancer (24), breast cancer (25), and esophageal carcinoma (26). miR485 has been shown to be relevant to the pathogenesis of breast cancer (27-29), gastric cancer (30, 31), bladder cancer (32), lung adenocarcinoma (33), liver cancer (34), prostate cancer (35), and hepatocellular carcinoma (36, 37). One interesting paradigm involving miR485 was a regulatory circuit implicating NF-YB regulation by miR-485-3p, which then lead to changes in expression of DNA topoisomerase II α , causing changes in responsiveness to topoisomerase inhibitors (38). These studies involved the use of both rhabdomyosarcoma Rh30 cells and the leukemia CEM/VM-1-5 cell line (38). With respect to miR595, regulation of the Sox7 transcription factor by mir595 supported proliferation of human glioblastoma cell lines (39). miR595 has also been implicated in the pathogenesis of ovarian cancer (40), neuroblastoma (41), and hepatocellular carcinoma (42).

We found, by comparing the transcriptomes of tumors from patients that survived greater or less than six months, that patients with diffuse intrinsic pontine glioma differentially express a series of non-coding RNA transcripts which include microRNAs and snoRNAs. Of ten differentially expressed ncRNA, the expression of all of these ncRNA were significantly lower in the tumors of patients that survived greater than 6 months save for one ncRNA transcript, SNORA22. The expression of SNORA22 alone was correlated in a linear fashion with the amount of time each patient survived (overall survival), and this correlation approached but did not meet statistical significance (95% confidence). All of the microRNA identified here as differentially expressed based on patient outcomes in DIPG have previously been implicated in cancer pathogenesis in a multitude of cancers. This is the first report to identify the differential expression of these microRNA based on patient outcomes in DIPG. These results merit further study into the role of each of these microRNA and snoRNA in the etiology of the DIPG, as well as if any can play a role in patient stratification at the time of diagnosis into groups of patients that will suffer from more severe disease courses and might

benefit from more intensive radiation therapy. Understanding the transcriptional landscape of this disease is the first step towards identification of therapeutic targets and the development of novel therapeutic modalities for patients with DIPG.

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Figure 1: miR939 is differentially expressed in the DIPG tumors based on survival greater or less than six months.

The expression of miR939 is graphically depicted (A-D) as dots representing the expression of miR939 in each individual patient with the horizontal black bar representing the mean (A); in a violin plot with the dotted horizontal black line representing the median (B); as a bar graph with the error bars denoting the standard error of the mean (C); and with each patient individual arrayed across the X axis (D). A statistical test was performed to evaluate the significance of the difference in expression values between each group of patients; the result of this test is shown as a p-value on each graph.

Figure 2: miR429 is differentially expressed in the DIPG tumors based on survival greater or less than six months.

The expression of miR429 is graphically depicted (A-D) as dots representing the expression of miR429 in each individual patient with the horizontal black bar representing the mean (A); in a violin plot with the dotted horizontal black line representing the median (B); as a bar graph with the error bars denoting the standard error of the mean (C); and with each patient individual arrayed across the X axis (D). A statistical test was performed to evaluate the significance of the difference in expression values between each group of patients; the result of this test is shown as a p-value on each graph.

Figure 3: miR595 is differentially expressed in the DIPG tumors based on survival greater or less than six months.

The expression of miR595 is graphically depicted (A-D) as dots representing the expression of miR595 in each individual patient with the horizontal black bar representing the mean (A); in a violin plot with the dotted horizontal black line representing the median (B); as a bar graph with the error bars denoting the standard error of the mean (C); and with each patient individual arrayed across the X axis (D). A statistical test was performed to evaluate the significance of the difference in expression values between each group of patients; the result of this test is shown as a p-value on each graph.

Figure 4: miR154 is differentially expressed in the DIPG tumors based on survival greater or less than six months.

The expression of miR154 is graphically depicted (A-D) as dots representing the expression of miR154 in each individual patient with the horizontal black bar representing the mean (A); in a violin plot with the dotted horizontal black line representing the median (B); as a bar graph with the error bars denoting the standard error of the mean (C); and with each patient individual arrayed across the X axis (D). A statistical test was performed to evaluate the significance of the difference in expression values between each group of patients; the result of this test is shown as a p-value on each graph.

Figure 5: miR485 is differentially expressed in the DIPG tumors based on survival greater or less than six months.

The expression of miR485 is graphically depicted (A-D) as dots representing the expression of miR485 in each individual patient with the horizontal black bar representing the mean (A); in a violin plot with the dotted horizontal black line representing the median (B); as a bar graph with the error bars denoting the standard error of the mean (C); and with each patient individual arrayed across the X axis (D). A statistical test was performed to evaluate the significance of the difference in expression values between each group of patients; the result of this test is shown as a p-value on each graph.

Figure 6: SNORD94 is differentially expressed in the DIPG tumors based on survival greater or less than six months.

The expression of SNORD94 is graphically depicted (A-D) as dots representing the expression of SNORD94 in each individual patient with the horizontal black bar representing the mean (A); in a violin plot with the dotted horizontal black line representing the median (B); as a bar graph with the error bars denoting the standard error of the mean (C); and with each patient individual arrayed across the X axis (D). A statistical test was performed to evaluate the significance of the difference in expression values between each group of patients; the result of this test is shown as a p-value on each graph.

Figure 7: SNORD65 is differentially expressed in the DIPG tumors based on survival greater or less than six months.

The expression of SNORD65 is graphically depicted (A-D) as dots representing the expression of SNORD65 in each individual patient with the horizontal black bar representing the mean (A); in a violin plot with the dotted horizontal black line representing the median (B); as a bar graph with the error bars denoting the standard error of the mean (C); and with each patient individual arrayed across the X axis (D). A statistical test was performed to evaluate the significance of the difference in expression values between each group of patients; the result of this test is shown as a p-value on each graph.

Figure 8: SNORD100 is differentially expressed in the DIPG tumors based on survival greater or less than six months.

The expression of SNORD100 is graphically depicted (A-D) as dots representing the expression of SNORD100 in each individual patient with the horizontal black bar representing the mean (A); in a violin plot with the dotted horizontal black line representing the median (B); as a bar graph with the error bars denoting the standard error of the mean (C); and with each patient individual arrayed across the X axis (D). A statistical test was performed to evaluate the significance of the difference in expression values between each group of patients; the result of this test is shown as a p-value on each graph.

Figure 9: SNORD49A is differentially expressed in the DIPG tumors based on survival greater or less than six months.

The expression of SNORD49A is graphically depicted (A-D) as dots representing the expression of SNORD49A in each individual patient with the horizontal black bar representing the mean (A); in a violin plot with the dotted horizontal black line representing the median (B); as a bar graph with the error bars denoting the standard error of the mean (C); and with each patient individual arrayed across the X axis (D). A statistical test was performed to evaluate the significance of the difference in expression values between each group of patients; the result of this test is shown as a p-value on each graph.

Figure 10: SNORD22 is differentially expressed in the DIPG tumors based on survival greater or less than six months.

The expression of SNORD22 is graphically depicted (A-D) as dots representing the expression of SNORD22 in each individual patient with the horizontal black bar representing the mean (A); in a violin plot with the dotted horizontal black line representing the median (B); as a bar graph with the error bars denoting the standard error of the mean (C); and with each patient individual arrayed across the X axis (D). A statistical test was performed to evaluate the significance of the difference in expression values between each group of patients; the result of this test is shown as a p-value on each graph.

Figure 11: Linear regression analysis shows lack of linear correlation between expression level of differentially expressed microRNAs and amount of time the patient survives in DIPG.

The expression of each microRNA on the X axis is graphed against the overall survival of each patient on the Y axis. A linear regression analysis was performed and the p-value assessing how linear and non-zero this correlation was is shown on each graph.

Figure 12: Linear regression analysis shows linear correlation between expression level of SNORD22 and amount of time the patient survives in DIPG.

The expression of each snoRNA on the X axis is graphed against the overall survival of each patient on the Y axis. A linear regression analysis was performed and the *p*-value assessing how linear and non-zero this correlation was is shown on each graph.

Table 1

Rank	Symbol	p-value	t	B	ID	GB_ACC
14	SNORD94	0.005574	-2.9451597	-4.51	ILMN_3245274	NR_004378.1
15	MIR939	0.005638	-2.9407319	-4.51	ILMN_3310371	NR_030635.1
29	MIR429	0.007442	-2.8332128	-4.52	ILMN_3309133	NR_029957.1
30	SNORD100	0.007554	-2.8273695	-4.52	ILMN_1673181	NR_002435.1
60	SNORA22	0.01233	2.6323121	-4.53	ILMN_3246713	NR_002961.1
65	SNORD65	0.0065	-2.8858855	-4.51	ILMN_3246353	NR_003054.1
69	MIR595	0.012938	-2.612779	-4.53	ILMN_3309879	NR_030325.1
140	MIR154	0.019527	-2.4423918	-4.54	ILMN_3308833	NR_029704.1
197	MIR485	0.023651	-2.3609477	-4.54	ILMN_3309764	NR_030160.1
200	SNORD49A	0.023731	-2.3594996	-4.54	ILMN_3248562	NR_002744.1

Table 1: Nine of the top 1% of differentially expressed genes when comparing the transcriptomes of those who survive greater or less than six months are non-coding RNA including snoRNA and microRNA.

The rank of expression out of 29285 total transcripts, the gene symbol, the *p*-value of differential expression globally, a moderated *t*-statistic (*t*), *B*, the log-odds of differential expression between both groups, the chromosome the gene is encoded on, the GenBank accession ID, as well as the probe ID from Illumina and Genbank accession code for each transcript are displayed in this table (all provided by GEO2R).