Iroquois transcription factors are perturbed in diffuse intrinsic pontine glioma.

Shahan Mamoor¹

¹Thomas Jefferson School of Law

mamoorsk@tjssl.edu

San Diego, CA 92101

The brain cancer diffuse intrinsic pontine glioma (DIPG) is the most fatal of all cancers with a 5-year survival rate of less than 1%, meaning greater than 99% of patients diagnosed with DIPG will expire within 5 years (1). Systems-level analyses of the cancer transcriptome compared to the tissue from which it arises presents a unique opportunity to gain insights into the transcriptional behavior of each cancer and how it differs from its tissue of origin (2). In this study I used published microarray data to compare the DIPG tumor transcriptomes to that of the normal brain (3). In both datasets, I found differential expression of an Iroquois transcription factor in DIPG tumors: in one dataset, IRX2, IRX5 and IRX3 were among the most differentially expressed genes in the tumors of patients with DIPG, and in a separate dataset, IRX4 was significantly differentially expressed when compared to control brain tissues. IRX proteins may be important molecules in the biology of diffuse intrinsic pontine gliomas.

Keyword: diffuse intrinsic pontine glioma, DIPG, comparative transcriptomics, systems oncology, differential gene expression, IRX2, IRX5, IRX3, IRX4, Iroquois transcription factors, brain cancer, brainstem glioma.

Introduction

Diffuse intrinsic pontine glioma is the most common tumor of the brainstem found in children, and is the deadliest cancer known to man (1, 4). In children and adolescents less than 14 years of age, gliomas represent 10-15% of all brain cancers. Gliomas are cancers of the glial cells (5), classically considered support cells to the nerve cells that conduct electrical signals in the brain (central nervous system) and throughout the entire body (peripheral nervous system). Broadly, the brain is anatomically divided into the cortex, cerebellum and brainstem (6). Of the gliomas diagnosed in the brainstem of children and adolescents, 80% are DIPG tumors. The only treatment with temporary benefit for DIPG is radiation therapy (7), with chemotherapeutic trials thus far proving of little use, even in transient benefit (8). There is a critical need for the discovery of novel pathways for therapeutic intervention in DIPG.

Systems-level analyses of cancers presents a unique opportunity for discovery of therapeutic targets and for molecular classification of poorly understood cancers (9). Previous systems level analyses have described DIPG as possessing three molecular subtypes, including silent, H3K27M, and MYCN, and found that 20% of patients diagnosed with DIPG contained an activating mutation in the activin receptor gene ACVR (3). This mutation lead to constitutive phosphorylation of SMAD, a TGF-beta pathway intermediate, and this caused expression of action pathway targets ID1 and ID2 (3), describing a paradigm by which the ID transcription factors were activated by gene mutation in patients with DIPG.

Differential gene expression permits for an unbiased assessment of the total transcription in any given state and how it differs most significantly to what it is being compared (7). In this study I compared the tumor transcriptomes of 35 patients diagnosed with DIPG to the transcriptomes of healthy brain tissue from 10 individuals (3) by global differential gene expression analysis using a published dataset. This analysis revealed that three members of the Iroquois transcription factor family, IRX2, IRX5 and IRX3 were among the top one hundred most differentially expressed genes, or the RNA messages whose level was most different between healthy brain and DIPG brain tumor. This was out of over 29,000

independent transcripts detected and measured (3). Iroquois transcription factors (10, 11) should be assessed for their ability to be targeted with small molecule inhibition, whether their inhibition would be harmful in healthy tissues, and whether any target genes of IRX2, IRX5, or IRX5 represent viable targets for therapeutic discovery in this consistently fatal disease.

Methods

Dataset GSE50021 (3) was utilized for this analysis, in conjunction with GEO2R, to perform differential gene expression analysis on healthy brain tissue (n=10) and tumor samples from patients diagnosed with DIPG (n=35). 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. GSE26576 was also used for this analysis, with n=2 normal brainstem tissue samples and n=2 DIPG pre-treatment tumor samples. The Benjamini and Hochberg method of p-value adjustment was used. Log transformation was set to “Auto-detect” and the submitter supplied category of platform annotation was used. For statistical analysis to compare the RNA expression values between tumor and control groups, an unpaired, two-tailed t-test with Welch’s correction was used (PRISM 8.1.2) (227).

Results

We performed global differential gene expression analysis in diffuse intrinsic pontine glioma, or DIPG by comparing the cancer transcriptome to the brain transcriptome in health, using microarray data from tumors from 35 patients with DIPG versus 10 control brain tissue samples. We found that three separate genes from the Iroquois family of transcription factors were among the genes whose expression was most different between control, non-cancerous brain tissue and the brain tumors of patients with DIPG (Table 1).

IRX2, IRX5, and IRX3 are among the most differentially expressed genes in the tumors of patients with DIPG when compared to healthy brain tissue.

IRX2 was the fifth most differentially expressed gene out of 29285 transcripts detected and measured by the microarray data analyzed in this study (Table 1; $p=6.77E-07$). IRX5 was the sixty-fourth most differentially expressed gene out of 29285 transcripts detected and measured by the microarray data analyzed in this study (Table 1; $p=5.42E-04$). IRX3 was the seventy-sixth most differentially expressed gene out of 29285 transcripts detected and measured by the microarray data analyzed in this study (Table 1; $p=7.2E-04$). Thus, three Iroquois transcription factors were represented among the genes discovered by systems-level comparative transcriptome analysis as having an expression level most significantly different from that of the tissue they arise from, the brain.

Expression of the Iroquois homeobox transcription factor genes IRX2, IRX5, and IRX3 is significantly higher in the tumors of patients with DIPG compared to healthy brain tissue.

IRX2, IRX5, and IRX3 were all expressed at significantly higher levels in the tumors of patients with DIPG than in control brain tissue (Figure 1A and Figure 1D: IRX2: $p<0.0001$; Figure 1B and Figure 1E: IRX5: $p=0.0015$; Figure 1C and Figure 1F: IRX3: $p=0.0004$). Thus, three independent Iroquois transcription factors were possessed significantly higher expression at the RNA level in the tumors of patients with DIPG as compared to control brain tissue.

IRX4 is differentially expressed in DIPG tumors.

We analyzed a separate microarray dataset generated using the tumor tissue of patients with DIPG, comparing the global gene expression profiles of normal brainstem to that of two DIPG tumors. We found that in this dataset, IRX4 was significantly differentially expressed in DIPG tumors. When sorting all transcripts measured by microarray based on change between DIPG tumors and the brainstem, IRX ranked 1012 out of 54675 total transcripts. Differential expression of IRX4 in DIPG tumors was statistically significant (Table 2; $p=0.0050279$).

Discussion

Diffuse intrinsic pontine glioma is the deadliest cancer known to man with a five-year survival rate of less than 1%, and a ten-year survival rate of 0%. The lethality of this disease demands a comprehensive understanding of the transcriptional behavior of the DIPG tumor and how it relates to the that of brain tissue in health. We performed such an analysis in this study, using published microarray data containing the transcriptome information of 35 tumors from patients diagnosed with DIPG compared to 10 samples of control, non-cancerous brain tissue. This analysis revealed that three Iroquois family transcription factors were among the most differentially expressed genes in the DIPG transcriptome compared to the brain transcriptome in health, and that the expression of IRX2, IRX5 and IRX3 were significantly higher in the tumors of patients with DIPG than in control brain tissue. In a separate microarray dataset, we observed significant differential expression of IRX4 in DIPG tumors.

Irx2 is involved in normal cerebellum formation in mice (12). One study found that Irx2 is expressed at higher levels in osteosarcoma cell lines and in the tumors of patients with osteosarcoma, and genetic inhibition of Irx2 *in vitro* caused a decrease in osteosarcoma cell proliferation (13). Higher expression of Irx2 was associated with a more aggressive disease course in osteosarcoma (13). Another study found that Irx2 was amplified in soft tissue sarcomas (14). Irx5 is required for establishing the polarization gradient in the murine heart via repression of potassium channel expression in the endocardial myocardium (15).

Irx5 is also required for the development of retinal cone bipolar cells in mice (16). In humans, mutations in Irx5 cause a recessive disorder causing defects in craniofacial development and in germ cell migration, and using *Xenopus* modeling, Bonnard et al. found that Irx5 was involved in the regulation of migration of progenitor cell populations to the branchial arches by repression of Sdf1 (17). Mechanistically, this was found to occur via interactions with two GATA zinc-finger proteins, GATA3 and TRPS (17). Irx5 is a downstream target of Hoxb4 (18). Moreover, repression of Irx5 was reported to be involved in small

intestine development, and this repression was found to be exerted by Id2 (19). With respect to Irx5 in tumorigenesis, one study found that Irx5 was associated with metastasis in colorectal cancer (20).

Irx3 has been found to have an antagonistic relationship with Irx5 in development of the proximal limbs and patterning of the anterior and proximal limb skeleton, and this was negatively regulated by Sonic hedgehog (Shh) signaling (21). Specially, formation of the tibia, femur, and the first digit was dependent on Irx3 and Irx5 (21), and Irx3/5 was required for specification of progenitors in the limb bud in this developmental process in mice (21). Irx3 is thought to be involved in the development of the kidneys in mice (22, 23). In Wilms tumor of the kidney, a sub-group of patients contain a deletion in 16q, and this region contains Irx3, Irx5, and Irx6 (24). Investigators of one recent study found that Irx3 and Irx5 had non-overlapping functions in Wilms tumor; Irx3/Irx5 double knockout mice displayed reduced nephron formation, and while Irx3^{-/-} mice possessed active canonical Wnt signaling, Irx5^{-/-} mice displayed activated non-canonical Wnt signaling (25). Mengelbier et al. suggested that activation of Irx3 signaling but inhibition of Irx5 signaling might be an effective strategy for therapeutic differentiation of Wilms' tumors (25). The expression of Irx3 was also found to be perturbed in hepatocellular carcinoma (26). Together, multiple studies have indicated a role for the Irx2, Irx5, and Irx3 transcription factors in cancers (13, 14, 20, 24, 26) but this is the first study to report differential and/or perturbed expression of Iroquois transcription factors in the diffuse intrinsic pontine glioma brainstem cancer.

We used unbiased, systems-level comparative transcriptomic study of a published microarray dataset from the tumors of patients with DIPG to perform global differential gene expression analysis. Specific analysis of IRX2, IRX5, IRX3 or IRX4 in brainstem development, in proliferation of human brainstem cells *in vitro*, and assessment of the effect of IRX2, IRX5, IRX3 or IRX4 genetic inhibition on tumor progression and maintenance in mouse models of DIPG (27-30) must be performed in light of the data presented here. IRX2, IRX5, IRX3 and/or IRX4 transcription factors could represent a novel therapeutic target, as could one or a

combination of their downstream targets. The aggressive nature of DIPG and total lack of chemotherapeutic options demands further study into the role of IRX transcription factors in the etiology and/or treatment of DIPG.

References

1. Vanan, M.I. and Eisenstat, D.D., 2015. DIPG in children—what can we learn from the past?. *Frontiers in oncology*, 5, p.237.
2. Zheng, P.Z., Wang, K.K., Zhang, Q.Y., Huang, Q.H., Du, Y.Z., Zhang, Q.H., Xiao, D.K., Shen, S.H., Imbeaud, S., Eveno, E. and Zhao, C.J., 2005. Systems analysis of transcriptome and proteome in retinoic acid/arsenic trioxide-induced cell differentiation/apoptosis of promyelocytic leukemia. *Proceedings of the National Academy of Sciences*, 102(21), pp. 7653-7658.
3. Buczkowicz, P., Hoeman, C., Rakopoulos, P., Pajovic, S., Letourneau, L., Dzamba, M., Morrison, A., Lewis, P., Bouffet, E., Bartels, U. and Zuccaro, J., 2014. Genomic analysis of diffuse intrinsic pontine gliomas identifies three molecular subgroups and recurrent activating ACVR1 mutations. *Nature genetics*, 46(5), p.451.
4. Grimm, S.A. and Chamberlain, M.C., 2013. Brainstem glioma: a review. *Current neurology and neuroscience reports*, 13(5), p.346.
5. Ohgaki, H. and Kleihues, P., 2005. Epidemiology and etiology of gliomas. *Acta neuropathologica*, 109(1), pp.93-108.
6. Salamon, G. and Huang, Y.P., 2012. *Radiologic anatomy of the brain*. Springer Science & Business Media.
7. Janssens, G.O., Jansen, M.H., Lauwers, S.J., Nowak, P.J., Oldenburger, F.R., Bouffet, E., Saran, F., Kamphuis-van Ulzen, K., van Lindert, E.J., Schieving, J.H. and Boterberg, T., 2013. Hypofractionation vs conventional radiation therapy for newly diagnosed diffuse intrinsic pontine glioma: a matched-cohort analysis. *International Journal of Radiation Oncology* Biology* Physics*, 85(2), pp.315-320.
8. Gwak, H.S. and Park, H.J., 2017. Developing chemotherapy for diffuse pontine intrinsic gliomas (DIPG). *Critical reviews in oncology/hematology*, 120, pp.111-119.
9. Liang, P. and Pardee, A.B., 2003. Analysing differential gene expression in cancer. *Nature Reviews Cancer*, 3(11), p.869.

10. Gómez-Skarmeta, J.L. and Modolell, J., 2002. Iroquois genes: genomic organization and function in vertebrate neural development. *Current opinion in genetics & development*, 12(4), pp.403-408.
11. Biloni, A., Craig, G., Hill, C. and McNeill, H., 2005. Iroquois transcription factors recognize a unique motif to mediate transcriptional repression in vivo. *Proceedings of the National Academy of Sciences*, 102(41), pp.14671-14676.
12. Matsumoto, K., Nishihara, S., Kamimura, M., Shiraishi, T., Ootoguro, T., Uehara, M., Maeda, Y., Ogura, K., Lumsden, A. and Ogura, T., 2004. The prepattern transcription factor *Irxd2*, a target of the FGF8/MAP kinase cascade, is involved in cerebellum formation. *Nature neuroscience*, 7(6), p.605.
13. Liu, T., Zhou, W., Zhang, F., Shi, G., Teng, H., Xiao, J. and Wang, Y., 2014. Knockdown of *IRX2* inhibits osteosarcoma cell proliferation and invasion by the AKT/MMP9 signaling pathway. *Molecular medicine reports*, 10(1), pp.169-174.
14. Adamowicz, M., Radlwimmer, B., Rieker, R.J., Mertens, D., Schwarzbach, M., Schraml, P., Benner, A., Lichter, P., Mechttersheimer, G. and Joos, S., 2006. Frequent amplifications and abundant expression of *TRIO*, *NKD2*, and *IRX2* in soft tissue sarcomas. *Genes, Chromosomes and Cancer*, 45(9), pp.829-838.
15. Costantini, D.L., Arruda, E.P., Agarwal, P., Kim, K.H., Zhu, Y., Zhu, W., Lebel, M., Cheng, C.W., Park, C.Y., Pierce, S.A. and Guerchicoff, A., 2005. The homeodomain transcription factor *Irxd5* establishes the mouse cardiac ventricular repolarization gradient. *Cell*, 123(2), pp.347-358.
16. Cheng, C.W., Chow, R.L., Lebel, M., Sakuma, R., Cheung, H.O.L., Thanabalasingham, V., Zhang, X., Bruneau, B.G., Birch, D.G., Hui, C.C. and McInnes, R.R., 2005. The Iroquois homeobox gene, *Irxd5*, is required for retinal cone bipolar cell development. *Developmental biology*, 287(1), pp.48-60.
17. Bonnard, C., Strobl, A.C., Shboul, M., Lee, H., Merriman, B., Nelson, S.F., Ababneh, O.H., Uz, E., Güran, T., Kayserili, H. and Hamamy, H., 2012. Mutations in *IRX5* impair craniofacial development and germ cell migration via *SDF1*. *Nature genetics*, 44(6), p.709.
18. Theokli, C., Morsi El-Kadi, A.S. and Morgan, R., 2003. TALE class homeodomain gene *Irxd5* is an immediate downstream target for *Hoxb4* transcriptional regulation. *Developmental dynamics: an official publication of the American Association of Anatomists*, 227(1), pp. 48-55.

19. Mori, K., Nakamura, H., Kurooka, H., Miyachi, H., Tamada, K., Sugai, M., Takumi, T. and Yokota, Y., 2018. Id2 determines intestinal identity through repression of the foregut transcription factor *Irx5*. *Molecular and cellular biology*, 38(9), pp.e00250-17.
20. Zhu, Q., Wu, Y., Yang, M., Wang, Z., Zhang, H., Jiang, X., Chen, M., Jin, T. and Wang, T., 2019. IRX5 promotes colorectal cancer metastasis by negatively regulating the core components of the RHOA pathway. *Molecular carcinogenesis*.
21. Li, D., Sakuma, R., Vakili, N.A., Mo, R., Puvion-Rodan, V., Deimling, S., Zhang, X., Hopyan, S. and Hui, C.C., 2014. Formation of proximal and anterior limb skeleton requires early function of *Irx3* and *Irx5* and is negatively regulated by Shh signaling. *Developmental cell*, 29(2), pp.233-240.
22. Houweling, A.C., Dildrop, R., Peters, T., Mummenhoff, J., Moorman, A.F., Rüther, U. and Christoffels, V.M., 2001. Gene and cluster-specific expression of the Iroquois family members during mouse development. *Mechanisms of development*, 107(1-2), pp.169-174.
23. Reggiani, L., Raciti, D., Airik, R., Kispert, A. and Brändli, A.W., 2007. The prepattern transcription factor *Irx3* directs nephron segment identity. *Genes & development*, 21(18), pp.2358-2370.
24. Mengelbier, Linda Holmquist, Jenny Karlsson, David Lindgren, Ingrid Øra, Margareth Isaksson, Ildiko Frigyesi, Attila Frigyesi, Johannes Bras, Bengt Sandstedt, and David Gisselsson. "Deletions of 16q in Wilms tumors localize to blastemal-anaplastic cells and are associated with reduced expression of the IRXB renal tubulogenesis gene cluster." *The American journal of pathology* 177, no. 5 (2010): 2609-2621.
25. Holmquist Mengelbier, L., Lindell-Munther, S., Yasui, H., Jansson, C., Esfandyari, J., Karlsson, J., Lau, K., Hui, C.C., Bexell, D., Hopyan, S. and Gisselsson, D., 2019. The Iroquois homeobox proteins IRX3 and IRX5 have distinct roles in Wilms tumour development and human nephrogenesis. *The Journal of pathology*, 247(1), pp.86-98.
26. Wang, P., Zhuang, C., Huang, D. and Xu, K., 2016. Downregulation of miR-377 contributes to IRX3 deregulation in hepatocellular carcinoma. *Oncology reports*, 36(1), pp.247-252.
27. Caretti, V., Zondervan, I., Meijer, D.H., Idema, S., Vos, W., Hamans, B., Bugiani, M., Hulleman, E., Wesseling, P., Vandertop, W.P. and Noske, D.P., 2011. Monitoring of tumor growth and post-irradiation recurrence in a diffuse intrinsic pontine glioma mouse model. *Brain Pathology*, 21(4), pp.441-451.
28. Cordero, F.J., Huang, Z., Grenier, C., He, X., Hu, G., McLendon, R.E., Murphy, S.K., Hashizume, R. and Becher, O.J., 2017. Histone H3. 3K27M represses p16 to accelerate

gliomagenesis in a murine model of DIPG. *Molecular Cancer Research*, 15(9), pp. 1243-1254.

29. Aoki, Y., Hashizume, R., Ozawa, T., Banerjee, A., Prados, M., James, C.D. and Gupta, N., 2012. An experimental xenograft mouse model of diffuse pontine glioma designed for therapeutic testing. *Journal of neuro-oncology*, 108(1), pp.29-35.
30. Kambhampati, M., Perez, J.P., Yadavilli, S., Saratsis, A.M., Hill, A.D., Ho, C.Y., Panditharatna, E., Markel, M., Packer, R.J. and Nazarian, J., 2015. A standardized autopsy procurement allows for the comprehensive study of DIPG biology. *Oncotarget*, 6(14), p. 12740.

Figure 1: Expression of the Iroquois homeobox transcription factor genes IRX2, IRX5, and IRX3 is significantly higher in the tumors of patients with DIPG compared to healthy brain tissue.

The expression of IRX2 (A, D), IRX5 (B, E), and IRX3 (C, F) in the tumors of patients with DIPG ($n=35$) and in healthy brain tissue ($n=10$) is graphically represented. In A, B, and C, each dot (blue = control, red = tumor) represents one tumor or control sample. In D, E, and F, the bars in the box and whisker plots represent the minimum and maximum value. In A, B, and C, the mean RNA expression value is denoted by the horizontal black bar.

Rank	Gene	p-value	t	B	Transcript	GB_ACC
5	IRX2	6.77E-07	5.56940646	4.91971	ILMN_14271	NM_033267.2
64	IRX5	5.42E-04	3.66063334	-0.38884	ILMN_27745	NM_005853.4
76	IRX3	7.2E-04	3.57091097	-0.61297	ILMN_4354	NM_024336.1

Table 1: IRX2, IRX5, and IRX3 are among the most differentially expressed genes in the tumors of patients with DIPG when compared to healthy brain tissue.

Rank order with respect to differential expression globally, gene symbol, *p*-value, *t* (a moderated *t*-statistic), *B*, the log-odds that the gene is differentially expressed between each group, transcript and Genbank accession ID are listed (provided by NCBI GEO2R).

Rank	ID	p-value	t	B	Gene	Gene name
1012	220225_at	0.0050279	-4.95	-1.531829	IRX4	iroquois homeobox 4

Table 2: Differential expression of IRX4 in DIPG tumors observed in an independent microarray dataset.

Rank order with respect to differential expression globally, probe ID, p -value, t (a moderated t -statistic), B , the log-odds that the gene is differentially expressed between each group, transcript and gene name are listed in this chart.