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14. ABSTRACT :
Diffuse intrinsic pontine glioma (DIPG) remains the most lethal childhood cancer. The slow progress in biological study and preclinical drug developmen is caused by the lack of fresh tumor tissues and clinically relevant animal models. The objective of our proposal is to determine if autopsied DIPG tissues can be used to develop orthotopic (intra-brain stem) xenograft mouse models that will replicate the biology of the original patient tumors. Through a series of studies, we demonstrated that a small percentage (approximately 10%) of DIPG tumor cells can survive the long-term post-mortem period of hypoxia/anoxia and starvation; and that the surviving tumor cells can form new xenograft tumors in the brain stems of SCID mice. We further demonstrated that the xenograft tumors replicate key histopathological features of the original patient tumors, and the cohort of animal models can be expanded through serial subtransplantations in vivo in mouse brain stems. We also completed the whole genome expression in 2 models and finished the whole exon sequencing in 4 DIPG tumors and identified a subset of novel dysregulated and mutated genes. Our results provide a new concept that can potentially revolutionize the use of autopsied tumor tissues for biological and preclinical studies of late/lethal stage of human cancers.

15. SUBJECT TERMS
DIPG, Orthotopic Xenograft, Mouse Model

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Introduction

Innovation: Clinically relevant animal models are desperately needed for diffuse intrinsic pontine glioma (DIPG), the most lethal childhood cancer that causes death in virtually all children with this disease within 1-2 years of diagnosis[1]. Because DIPGs are not amenable for surgery due to its location and infiltrative nature, autopsy provides the only and the most precious opportunity to collect tumor tissues for biological studies[2-4]. The objective of our proposal is therefore to determine if autopsied DIPG tissues can be used to develop orthotopic (intra-brain stem) xenograft mouse models that will replicate the biology of the original patient tumors. This highly innovative concept has not been tested before. A series of experiments will be performed to challenge two accepted paradigms: ***i)*** few or no tumor cells can survive in the autopsied tumor tissues, and it is impossible to harvest viable and tumorigenic tumor cells after the long-term (several to more than 24 hr) postmortem period of severe hypoxia/anoxia and starvation; ***ii)*** mouse brain stem is an extremely delicate and vital organ, and cannot be injected with DIPG tumor cells to create truly orthotopic xenograft models because surgical injuries will cause severe neurological deficit or even death – as has been well established in human beings – in the host animals.

Hypothesis and Specific Aims: Our central hypothesis is that a small population of DIPG cells possess the extraordinary survival and tumorigenic capacities that can survive the postmortem period of severe hypoxia and starvation, and regain their tumorigenic capabilities upon being returned to an appropriate microenvironment. Our 2nd hypothesis is that the resultant xenograft tumors will replicate the key biological features of the original patient DIPGs. This is because many of the viable tumor cells harvested from the autopsied DIPG tissues expressed known markers of cancer stem cells, which are shown to possess exclusive self-renewal and multipotent capacities to repopulate new tumors[5]. Our 3rd hypothesis is that the integrated genetic analysis of the xenograft tumors should lead to the identification of novel diagnostic markers and therapeutic targets.

To test these hypotheses, we propose the following **Specific Aims:**

1. To develop a panel of orthotopic xenograft models for pediatric DIPG through direct engraftment of autopsy tumor cells into the brain stems of Rag2/SCID mice.

2. To confirm that the resultant intra-brain stem xenograft tumors replicate the histopathological and invasive growth of the original patient tumors.

3. To identify new therapeutic targets in the xenograft tumors through comprehensive whole genome analysis of RNA and DNA.

Impact: We expected to collect 5-10 additional autopsied DIPGs in the funding year. Since our preliminary data showed that 50% autopsied tumors were tumorigenic, our anticipated total number of tumor formation would be 4-7 (50% of the 9-14 tumors). Completion of our proposed study will ***i***) provide a set of critically needed and clinically relevant animal models for DIPG and ***ii***) identify series of novel therapeutic targets. More importantly, our findings will also provide a new concept that can potentially revolutionize the use of autopsied tumor tissues for biological and preclinical studies of late/lethal stage of human cancers by demonstrating that the viable and tumorigenic tumor cells can be harvested from postmortem tissues.

Furthermore, our study can potentially fine-tune the cancer stem cell hypothesis and identify the ultimate seed cells of human cancers, which can be defined as the tumor cell that ***i***) are clinically proven to be therapy-resistant; ***ii***) are the direct cause of patients' death; and ***iii***) have demonstrated extraordinary survival and tumorigenic capabilities. Subsequent efforts aimed at understanding their mechanism(s) of drug-resistance and survival should open a completely new paradigm for late/lethal phase of human cancers. Children in military families will directly benefit from our proposed study.

BODY

Specific Aim 1*: To develop a panel of orthotopic xenograft models for pediatric DIPG through direct engraftment of autopsy tumor cells into the brain stems of Rag2/SCID mice.* During the funding period, we have completed the following tasks:

1a. *Collection of five autopsied DIPG tumor tissues nation-wide:* A total of 5 new autopsied DIPG tumor tissues were procured. All the tumor tissues were harvested during the consented autopsy through meticulous collaborations with the families, physicians and pathologists. Three of the five tumors were collected from hospitals outside Houston (**Table 1**).

1b*. Evaluation of the relative abundances and the harvest of viable tumor cells for immediate intra-brain stem injection in Rag2/SCID mice:* In all the five samples, viable tumor cells, ranging from 1 x 10^6 ~ 10 x 10^6 cells, were harvested, followed by immediate injection (5 x 10^4 cells in 1 uL medium) into the brain stems of immune-deficient SCID mice (**Figure 2**). We have further optimized our surgical procedure to ensure safe and reproducible injection of tumor cells into mouse brain stems. The surgical related animal death was observed in 3 of the 121 (2.4%) animals that received the initial implantation of autopsied tumor cells, demonstrating the safety of our procedure. Tumor growth has since been monitored by MRI scanning and by the signs of neurological deficit. In two of the five models, tumor formation (defined as passage 1) has been noted. The xenograft tumor cells have been harvested and subtransplanted to expand the cohorts of mouse models (**Table 1**).

The evaluation of tumor cell growth in vitro: Established cell cultures can facilitate the initial biological and preclinical drug screenings. Since there is a lack of well established cell cultures of DIPGs, we tried to initiate cells cultures using all the autopsied tumor tissues and established xenograft tumors. In addition to traditional serum-based culture medium, we also used serum-free media (supplemented with different growth factors) that are shown to favor the growth of cancer stem cells. However, most of the autopsied DIPG tumor cells were not able to survive and proliferate *in vitro*, and the xenograft tumor cells appeared to have slightly better adaption to the *in vitro* culture conditions. During the funding period, we were able to propagate neurosphere lines from two xenograft tumors. There is a third line that just showed signs of growth as attached cells (**Table 2**). We are actively expanding these cells in culture.

1c. *Determination of the identity of viable tumor cells through comprehensive profiling of cancer stem cell surface markers:* The relative abundance of putative cancer stem cells were analyzed through fluorescence activate cell sorting (FACS) using a series of fluorochrome-conjugated and human-specific antibodies, including CD133, CD15, CD57, CD24, CD44 and CD117[6-18]. Variable levels of DIPG tumor cells expressing CD133, CD15, CD57 and CD24 were detected in the five tumors analyzed, ranging from trace amount (0.1%) up to 35%. Although CD133 and CD15 have been successfully utilized to isolate human malignant glioma stem cells, they were detected in only one of the five DIPGs. Cells expressing CD24 and CD44 were low as well. Two of the tumors expressed CD117, but the overall levels were low (<2%). CD57+ cells, however, were detected in all the five tumors analyzed and the relative abundance was much higher (5% to 35%) than the rest of markers (**Figure 2**). This finding suggested that CD57+ DIPG cells might have stronger survival capabilities than those established brain tumor stem cells (CD133+ and CD15+ cells) and warrants further functional validation as novel therapeutic target cells.

Specific Aim 2: *To confirm that the resultant intra-brain stem xenograft tumors replicate the histopathological and invasive growth of the original patient tumors.* We have completed the following tasks:

2a. *Examination of histological and immunohistochemical phenotypes and invasive growth patterns of the established xenograft tumors:* Characterization of the xenograft tumors was focused on the established models. During the funding period, we have completed the histopathological analysis of two models (IBs-1215DIPG and IBs-w0128DIPG) (**Figure 3**). In addition to H&E staining, whole mouse brains bearing intra-brain stem xenograft tumors were harvested at different passages (I, II and III) and paraffin

embedded. The serially sections were then subjected to immunohistochemical staining using a comprehensive series of antibodies to examine the species identity (human-specific antibodies against mitochondria), cell proliferation (Ki-67), glial differentiation (GFAP), neuronal differentiation (SYN and MAP), angiogenesis (vWF), intermediate neurofilament (Vimentin), as well as genes involved in the regulation of stem cells self-renewal (Nestin, BMI-1, SOX2). Our results confirmed that the xenograft tumors were derived from the implanted human DIPG cells. They were highly invasive and very active in cell proliferation. The xenograft tumor cells expressed glial marker GFAP and were strongly positive for stem cell markers (Nestin, BMI-1 and SOX2) (**Figure 3**). Whole mouse brains from the remaining tumorigenic models have been harvested and paraffin embedded. Once the serially sections (150-250 sections/brain) of these brains are completed, they will be subjected to the histopathological examinations as described above.

2b. S*erial subtransplantation of xenograft tumors in vivo in mouse brain stems:* This was performed in the two newly established models and the six models that were implanted before the initiation of the current funding period but were still at the early passages of intra-brain stem growth (**Table 2**). Our goal is to pass them for at least five times to demonstrate their capability of sustained growth in vivo in mouse brains as some of these model may fail to repopulation new tumors after several passages. Repeated long-term subtransplantation is also required to confirm that the key genetic abnormalities are stably maintained during the serial subtransplantations. Two of the eight models have recently entered the fifth and the sixth passage, respectively. The total number of animals used during the funding period, including the strain information and the mouse number for each model, was summarized in **Table 3**.

Specific Aim 3: *To identify new therapeutic targets in the xenograft tumors through comprehensive whole genome analysis of RNA and DNA*. We performed whole genome gene expression profiling (to identify abnormally expressed genes) and whole exome sequencing (to detect mutated genes).

3a. W*hole genome gene expression profiling:* This is completed in two sets of DIPG models (including patient tumor and xenograft tumors at passage I, II, and III) using RNA from normal brain stem and/or pooled normal brain tissues as references**.** A database containing the expression levels of whole human genome has been constructed, from which the differentially expresses genes can be easily identified. This database was also designed as an open platform so that new data from additional DIPG models can be smoothly incorporated into it. For the remaining models that are still at the early passages, we have systemically snap-frozen fresh xenograft tumor tissues. Once the xenograft tumors have been passaged for more than five times, xenograft tumors from passage I, III and V will be retrieved, and the total RNAs extracted using the identical assays. These samples will then be subjected to gene expression profiling using the same batch of arrays to minimize batch differences. We will seek additional funding to complete the remaining models.

3b. *Whole genome genotyping and complete exon sequencing:* This has been completed through whole exon sequencing in four of the autopsied DIPG tumor tissues, in which both tumor and normal DNAs (of high quality) were available, through collaboration with Dr. Charles Keller at Oregon Health Science University. The exon sequencing was partially supported by the Cure Starts Now Foundation. In all four DIPGs, we identified mutated genes, ranging from 12 in C1220DIPG to 47 in W0128DIPG (27.75 ± 16.1 genes), per tumor (**Figure 4**). Many of the mutated genes are novel and have not been described previously. Except for W0128DIPG, we also found additional genes with small insertion or deletion (referred to as indel in **Figure 3**) in the remaining three tumor (**Figure 4**). The overall number of genes involved, however, was small, from 2 genes to 6 genes. We are now in the process of validating these DNA mutations in the corresponding xenograft tumors. Since all these patients have been previously treated with chemo- and radiation, it is possible that some of the mutations/insertions/deletions were therapy-related and may have played a role in tumor recurrence.

KEY RESEARCH ACCOMPLISHMENTS

- Confirmed the existence of viable and tumorigenic tumor cells in autopsied DIPG tumor tissues:
  - Demonstrated that a small percentage (approximately 10%) of DIPG tumor cells can survive the long-term post-mortem period of hypoxia/anoxia and starvation.
  - Demonstrated that the surviving tumor cells were tumorigenic, and can form new xenograft tumors the brain stems of SCID mice.

- Demonstrated that the xenograft tumors replicate key histopathological features of the original patient tumors, and the cohort of animal models can be expanded through subtransplantations.

- Completed the whole genome expression in 2 models and finished the whole exon sequencing in 4 DIPG tumors, identifying novel dysregulated and mutated genes.

REPORTABLE OUTCOMES

Abstract

Patricia A. Baxter Zhigang Liu, Xiumei Zhao, Yujing Zhang, Jack MF Su, Adekunle Adesina, Murali Chintagumpala, Ching C. Lau, Susan M. Blaney, Xiao-Nan Li. *Identification of therapy resistant pediatric brain tumor cells that have extraordinary survival and tumorigenic capabilities: the ultimate seed cells that drives the growth of autopsied tumor cells in mouse brains.* Presented at AACR Annual Meeting 2012 (abstract #2364)

CONCLUSIONS

In this study, we have completed the proposed experiments and successfully tested our hypotheses. Our results provided strong experimental evidence to demonstrate the feasibility of utilizing autopsied tumor tissues for the development of orthotopic xenograft mouse models. We have provided a set of critically needed and clinically relevant animal models for DIPGs, and identified multiple genetic abnormalities that warrants further validation as novel therapeutic targets. More importantly, our results provide a new concept that can potentially revolutionize the use of autopsied tumor tissues for biological and preclinical studies of late/lethal stage of human cancers.

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APPENDICES

A manuscript describing our findings is under preparation.

SUPPORTING DATA

Table 1. Summary of the autopsied DIPG tumors.

No.	Tumor ID	Age (yr)	Gender	Final Diagnosis	Patient location	Time Lapse*	Cell Viability
1	P1215†	7	Male	DIPG (autopsy)	Local	6	4.80%
2	W0128†	8	Male	DIPG (autopsy)	Local	17	9%
3	A1024†	7	Female	DIPG (autopsy)	Local	14	12.60%
4	AB10206†	13	Female	DIPG (autopsy)	~1100 miles	66	(*1.2 million*)**
5	J1204†	12	Female	DIPG (autopsy)	~1,500 miles	49	22.10%
6	C1220†	12	Male	DIPG (autopsy)	~1,1100 miles	77	(*0.6 million*)**
7	**D0227‡**	6	Male	DIPG (autopsy)	~400 miles	27	5.3
8	**A0417‡**	6	Female	DIPG (autopsy)	~400 miles	24	2.3
9	**A0518‡**	13	Female	DIPG (autopsy)	local	6	30%
10	**J0531‡**	14	Male	DIPG (autopsy)	~1,500 miles	40	(1.5 million)**
11	**H0712‡**	8	Female	DIPG (autopsy)	local	19	37%

Note: † Tumor tissues previously collected; ‡ Newly procured autopsy tumor tissues during the funding period. *Time lapse: from patient's death to the tumor processing; ** In these samples, most of the dead cells were already lysed, making it difficult to estimate the cell viability. Only the vial cells were counted.

Table 2. Growth of the autopsied DIPG cells in vivo and in vitro.

No.	Model ID	Tumorigenic in SCID mice?	Current Passage in Mouse Brain	Patient Tumor Cell Growth in Culture?	Xenograft Tumor Cell Growth in culture?
1	IBs-P1215DIPG*	Yes	4	No	Yes, neurosphere
2	IBs-W0128DIPG	Yes	4	Yes, short term	No
3	IBs-A1024DIPG	Yes	2	No	No
4	IBs-A10206DIPG	Yes	3	No	No
5	IBs-J1204DIPG	Yes	6	No	No
6	IBs-C1220DIPG	Yes	5	No	Yes, as attached
7	**IBs-D0227DIPG****	Yes	3	No	On-going
8	**IBs-A0417DIPG****	Pending	1	No	Not tested
9	**IBs-A0518DIPG****	yes	2	Yes, as neurosphere	Not tested
10	**IBs-J0531DIPG****	Pending	1	No	Not tested
11	**IBs-H0721DIPG****	Pending	1	On-going	Not tested

Note: * IBs= Intra-brain Stem; ** Indicates the models newly initiated during the funding year 2011-2012.

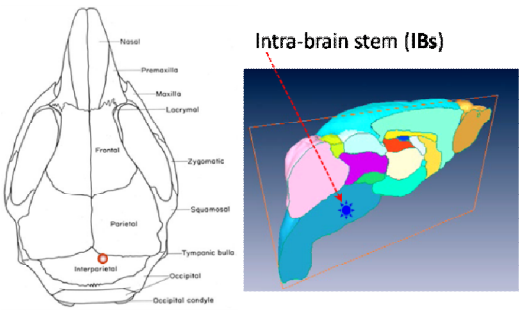

Figure 1. Images showing the location of blur hole (red dot in the left panel) and the location of the injected tumor cells in mouse brain stem. Tumor cells were resuspended in culture medium and injected 5.2 mm deep (from the cranial bone surface) into the middle of the mouse pon.

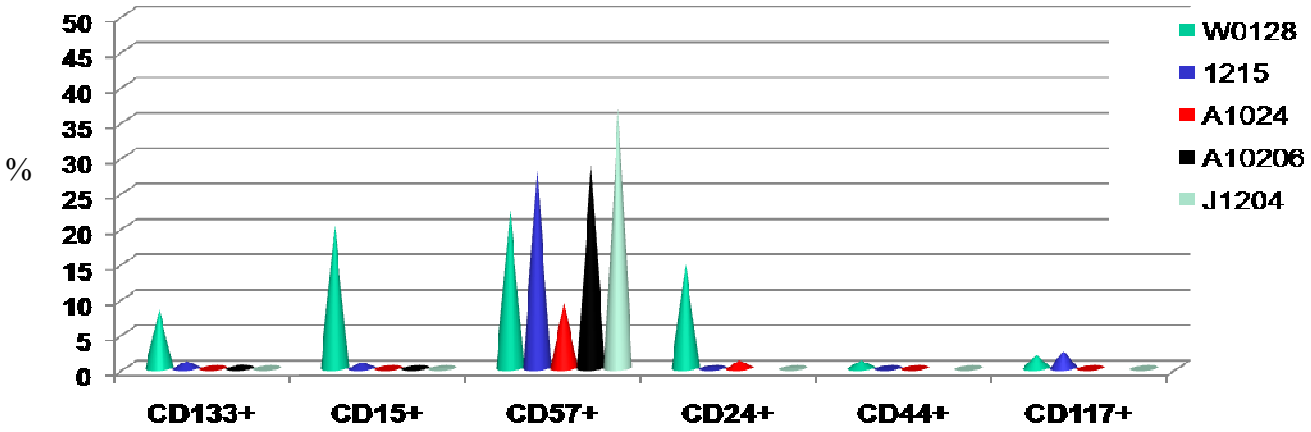

Figure 2. Graph showing the quantitative analysis of DIPG tumor cells expressing the putative cancer stem cell markers. Florochrome conjugated human-specific antibodies were incubated with single DIPG tumor cells and analyzed through fluorescence activated cell sorting (FACS). Dead cells and cell debris were gated out though PI staining.

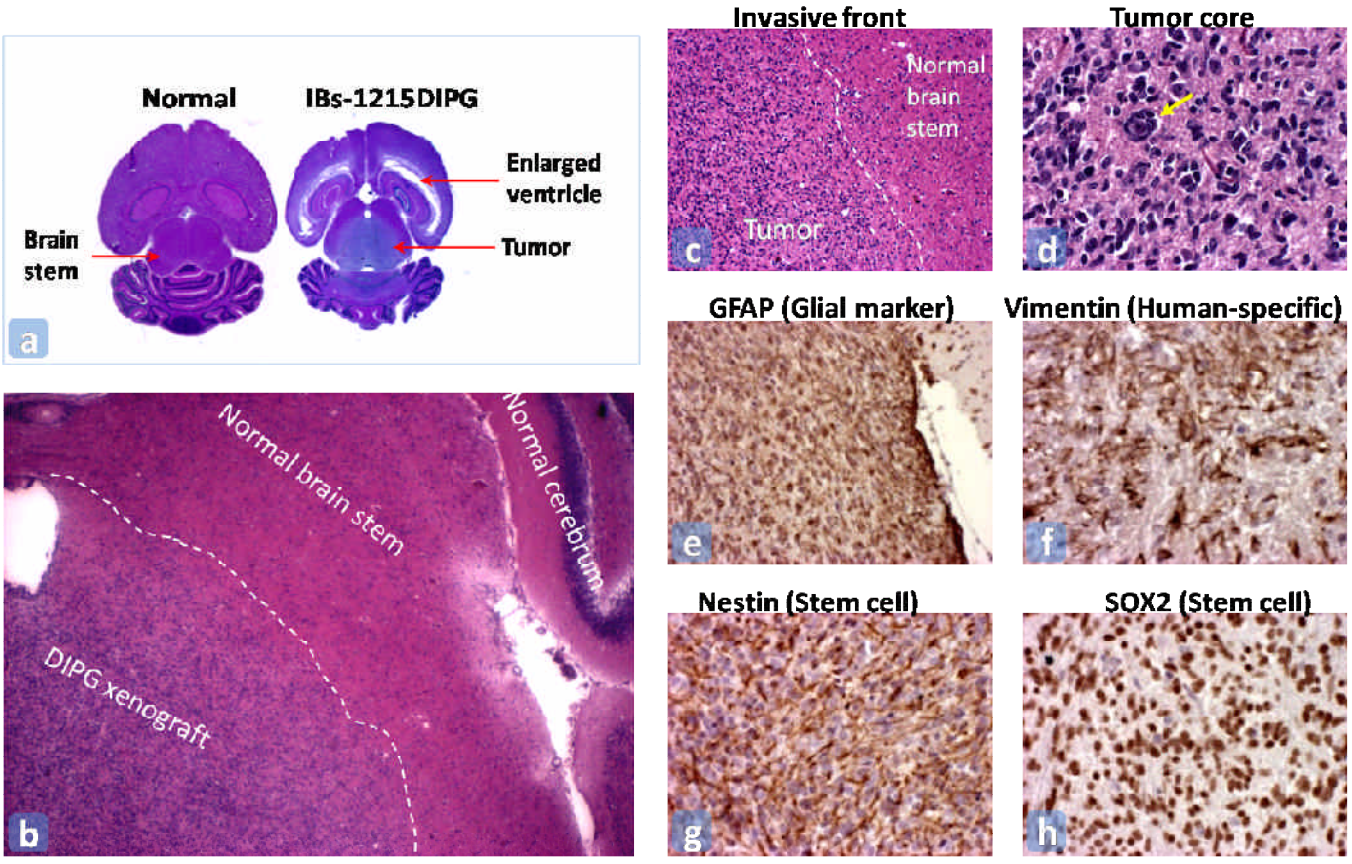

Figure 3. Representative images showing the intra-brain stem growth of DIPG xenograft tumor (IBs-P1215DIPG. ***a.*** Cross section of whole mouse brain showing the orthotopic xenograft tumor in mouse brain stem, causing hydrocephalus (enlarged left ventricle). The normal mouse brain image was placed on the left for comparison. ***b-d.*** H&E staining showing the microscopic morphology and the invasive growth of the established DIPG xenograft tumor. ***e-h.*** Immunohistochemical staining showing the expression of glial marker GFAP, human-specific neurofilament vimentin, and cancer stem cell marker Nestin and SOX2.

Table 3. Strain and number of animals used in the funded study.

No.	Model ID	Strain	Mouse Number
1	IBs-P1215DIPG	SCID	48
2	IBs-W0128DIPG	SCID	20
3	IBs-A1024DIPG	SCID	10
4	IBs-A10206DIPG	SCID	20
5	IBs-J1204DIPG	SCID	40
6	IBs-C1220DIPG	SCID	138
7	IBs-D0227DIPG	SCID	55
8	IBs-A0417DIPG	SCID	30
9	IBs-A0518DIPG	SCID	35
10	IBs-J0531DIPG	SCID	15
11	IBs-H0721DIPG	SCID	20
	Total	*SCID*	*431*

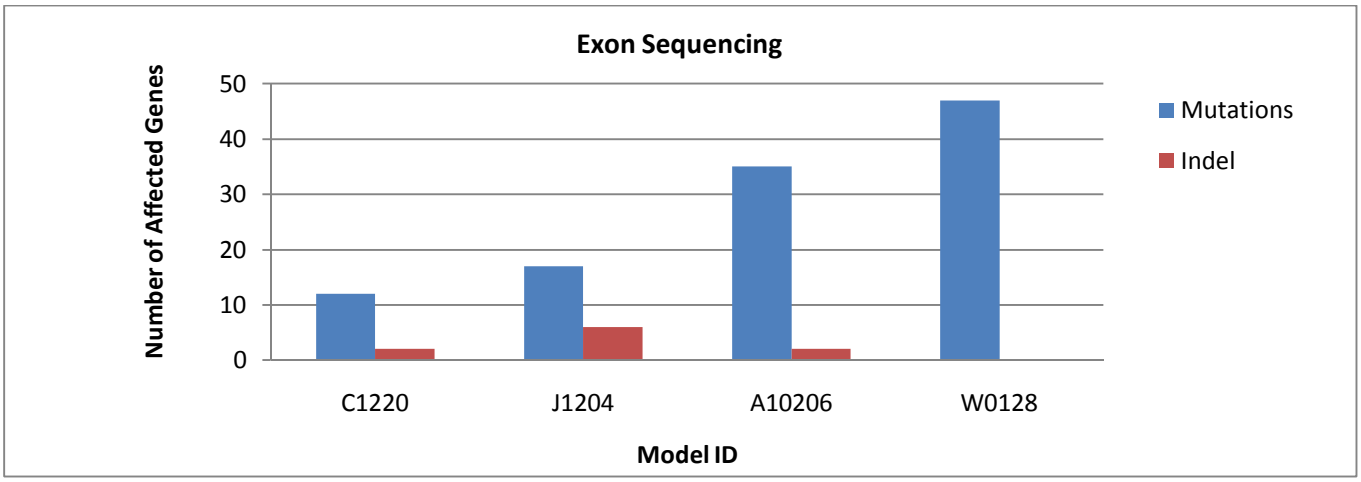

Figure 4. Graphs showing the total number of genes with mutation or indel (either insertion or deletion of small sequences) in the four DIPG tumors using matched normal DNA as reference.