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Exome Sequencing on Malignant Meningiomas Identified Mutations in Neurofibromatosis Type 2 (NF2) and Meningioma 1 (MN1) Genes

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Disclosure

X.Z., Y.L., F.W., and H.Z. are employees of BGI, a sequencing service company. K.W. is board member and shareholder of Tute Genomics, a bioinformatics software company.

Author Contributions

X.Z. and K.W. conceived and designed the study. X.Z., Y.L., and F.W. performed exome sequencing experiments, candidate gene sequencing experiments, and Sanger validation. J.H. and C.D. performed data analysis. H.J., J.H., Z.W., and L.W. provided materials and samples. X.Z., H.J., C.D., and K.W. drafted the manuscript. All authors read and approved the manuscript.

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Abstract

Background—Meningiomas are tumors originating from the membranous layers surrounding the central nervous system, and are generally regarded as "benign" tumors of the brain. Malignant meningiomas are rare and are typically associated with a higher risk of local tumor recurrence and a poorer prognosis (median survival time <2 years). Previous genome-wide association studies and exome sequencing studies have identified genes that play a role in susceptibility to meningiomas, but these studies did not focus specifically on malignant tumors.

Methods—We performed exome sequencing on five malignant meningiomas on the Illumina HiSeq2000 platform using Agilent SureSelect Human All Exon kits. We used wANNOVAR web server to annotate and prioritize variants, identified candidate genes with recurrent mutations, and validated selected mutations by Sanger sequencing. We next designed custom NimbleGen targeted region arrays on five candidate genes, and sequenced four additional malignant meningiomas.

Results—From exome sequencing data, we identified several frequently mutated genes including *NF2*, *MN1*, *ARID1B*, *SEMA4D*, and *MUC2*, with private mutations in tumors. We sequenced these genes in four additional samples and identified potential driver mutations in *NF2* (neurofibromatosis type 2) and *MN1* (meningioma 1).

Conclusions—We confirmed that mutations in *NF2* may play a role in progression of meningiomas, and nominated *MN1* as a candidate gene for malignant transformation of meningiomas. Our sample size is limited by the extreme rarity of malignant meningiomas, but our study represents one of the first sequencing studies focusing on the malignant subtype. [*Discovery Medicine 18(101):301-311, December 2014]*

Introduction

Meningiomas are the most common tumors arising from the meninges, the membranous layers surrounding brain and spinal cord, accounting for 34.7% of all Central Nervous System (CNS) tumors with an annual incidence rate of 60 per million individuals (Whittle *et al.*, 2004). Meningiomas are generally benign. Indeed, 80% of meningiomas are classified as benign (grade I), whereas 15%-20% are classified as atypical (grade II), and only 1-3% are malignant (grade III) according to World Health Organization (WHO) histological grading criteria (Louis *et al.*, 2007; Ryken and Chamberlain, 2011). Rare as they are, malignant meningiomas constitute a rather poor prognosis subtype, with recurrence rate up to 50-80% after surgical resection and with median survival of 1.5 years (Perry *et al.*, 1999). Therefore, to provide important insights into diagnosis and individualized treatment strategies for this aggressive tumor subtype, further understanding of its genetic basis is needed.

The genetics of meningiomas has been studied less extensively, compared to other more prevalent/complex cancers. Over the past few decades, some genetic studies such as

cytogenetic studies, linkage studies, and genome-wide association studies (GWAS) have suggested candidate genes and pathways that may play a role in meningioma progression (Pham *et al.*, 2011). For example, tumor suppressor genes, such as *NF2, DAL-1, CDKN2A, CDKN2B,* and various tissue inhibitors of matrix metalloproteinases (*TIMPs*), have been reported to be associated with tumor progression of meningiomas (Fernandez *et al.*, 1999; Ferner, 2007; Ferrara *et al.*, 2003; Gutmann *et al.*, 2000; Jagannathan *et al.*, 2008). Oncogenes such as *C-sis, c-myc, c-fos, Ha-ras, c-mos, TP73, bcl-2,* and *STAT3* have been noted to have a relatively high incidence of somatic mutations in meningiomas (Abramovich and Prayson, 2000; Carstens *et al.*, 1988; Detta *et al.*, 1993; Kazumoto *et al.*, 1990). To date, perhaps the most significant genetic finding on somatic mutations was still the disruption of tumor suppressor *NF2* on chromosome 22q12, which was confirmed to be a critical initiating event in the formation of approximately half of all meningiomas (Choy *et al.*, 2011; Pham *et al.*, 2011). Fewer studies have been conducted on germline mutations; however, a GWAS study of 859 meningioma patients and 704 controls identified *MLLT10* gene in a susceptibility locus at 10p12.31, with strong significance but moderate effect size ($P=1.88\times10^{-14}$, OR=1.46) (Dobbins *et al.*, 2011).

Recently, the advent of next-generation sequencing techniques made it possible to identify candidate genes and pathways in a larger scale, spurring the discovery of a spectrum of candidate genes, such as *SUFU* (Aavikko *et al.*, 2012), *SMO* (Brastianos *et al.*, 2013; Clark *et al.*, 2013), and *TRAF7* (Reuss *et al.*, 2013). However, such studies focused on analyzing benign meningiomas, which are much easier to sample due to its higher prevalence, while to a lesser extent exploring the most deadly malignant subtype that constitutes a major therapeutic challenge. For example, none malignant tumor was sequenced among 300 meningiomas used in a recent large-scale genomic study (Clark *et al.*, 2013). Another recent study did not include any malignant meningiomas for whole genome/exome sequencing in the discovery set, but included 3 malignant meningiomas for candidate gene sequencing in the extrapolation set (Brastianos *et al.*, 2013). Therefore, the crucial genetic alterations involved in malignant transformation still remained largely unknown.

To investigate the molecular genetics of malignant meningiomas, we have initiated a series of pilot studies that aimed at extensively identifying distinctive traits of malignant meningiomas from genomic, epigenomic, and transcriptome aspects. For example, on genomic level, a gene fusion event induced by local chromosomal inversion was recently pinpointed using high-throughput RNA-Seq technology (Gao *et al.*, 2013a). On epigenomic level, genes with hypermethylated CpG islands in malignant meningiomas (such as *HOXA6* and *HOXA9*) were found to coincide with the binding sites of polycomb repressive complexes (PRC) in early developmental stages, suggesting differentially methylated genes as potential diagnostic biomarkers or candidate causal genes for malignant transformation (Gao *et al.*, 2013b). On transcriptome level, 23 coexpression modules including a distinctive repression of meningioma tumor suppressor *MN1* were established from the weighted gene coexpression network analysis on transcriptome copy number alterations of malignant meningiomas (Chang *et al.*, 2013). Such studies paved the way for further investigation of malignant meningiomas, and a higher resolution of genomic scan in malignant meningiomas is entailed to complete the catalog of genetic mutations for this aggressive tumor subtype.

In the current study, we explored the genomic changes in malignant meningiomas using Whole Exome Sequencing (WES) to complement previous studies on benign ones. On five malignant meningiomas that we sequenced, we identified five genes (*NF2*, *MN1*, *ARID1B*, *SEMA4D*, and *MUC2*) that carry novel protein-altering variations and may be associated with progression of meningiomas. We also validated the candidate genes on an additional cohort of patients using targeted region sequencing on these five genes, further nominating *NF2* and *MN1* as candidate genes associated with malignant meningiomas. Our study represents the first such study focusing on malignant meningiomas, and provides genetic findings for future validation on this rare and deadly cancer.

Materials and Methods

Patient samples

After evaluating detailed history of pathology, availability of tissue specimens, and availability of high-quality DNAs, our study included nine patients with malignant meningiomas, including four males and five females. All enrolled patients in the registry came from Sun Yat-Sen University Cancer Center with written informed consents. Demographic and baseline clinical characteristics of these patients were examined manually and were shown in Table 1.

Library preparation

DNA samples were extracted from the tumor FFPE slides from these patients using QIAamp DNA FFPE kit (Qiagen, Valencia, CA, USA) according to the standard protocol. Samples with main DNA fragment larger than 250 bp were chosen for constructing exome sequencing library. For each sample, 2 μg purified genomic DNA was extracted and randomly fragmented using Covaris E210 to generate DNA fragments around 200 to 300 bp and then validated using 2% agarose gel. Next, we purified DNA fragments using AMpure XP beads (Beckman, Brea, CA, USA).

The fragmented DNA was treated with T4 polynucleotide kinase, T4 DNA polymerase, and Klenow fragment with dNTPs to create phosphorylated blunt termini. Then the end-repaired DNA fragments were incubated with Klenow fragment exo- and dATP to create 3′ over-hangs and were ligated to synthetic general adaptors.

Adaptor ligated-DNA fragments were amplified by ligation-mediated polymerase chain reaction (LM-PCR) and purified using AMpure XP beads. PCR products from Patients 1, 2, 3, 4, and 5 were hybridized to the Agilent SureSelect Human All Exon array (http://www.genomics.agilent.com/en/SureSelect-DNA-RNA/SureSelect-Human-All-Exon-Kits/?cid=AG-PT-177&tabId=AG-PR-1204) to prepare for exome sequencing of these 5 patients. As for the other 4 patients, we applied purified PCR product to NimbleGen targeted region array (see below) for enrichment to prepare for targeted region sequencing. Both non-captured and captured LM-PCR products were subjected to quantitative PCR to estimate the magnitude of enrichment. At last, the captured DNA was amplified to create enough fragments for sequencing.

Exome sequencing

Each captured library by Agilent SureSelect Human All Exon array was loaded on Hiseq2000 platform for sequencing. High-throughput sequencing was performed and the raw image files were processed by Illumina base calling software 1.7 for base calling with default parameters and the sequences of each library are generated as 90 bp paired-end reads. Adequate depth and coverage was achieved for each sample and therefore data from all five samples were used for further analysis.

Candidate region sequencing

As for candidate region sequencing of the five candidate genes, we designed the candidate region array using NimbleGen Design (http://www.nimblegen.com/products/nimbledesign/index.html), with following parameters: Preferred Close Matches-3, Maximum Close Matches-4, and final Target Bases Covered being 90.9%. We obtained the following candidate region: 29985287-30108845 and 28136281-28205468 for chromosome 22, 157034136-157596839 for chromosome 6, 91957869-92112446 for chromosome 9, and 1070443-1108847 for chromosome 11.

Next, similar as before, we subjected each library captured by NimbleGen targeted region arrays to Hiseq2000 platform for sequencing. High-throughput sequencing was performed and the raw image files were processed by Illumina base calling software 1.7 for base calling with default parameters and the sequences of each library are generated as 90 bp paired-end reads. Adequate depth and coverage was achieved for each sample and therefore data from all four samples were used for further analysis.

Variant calling

We used SeqMule (http://seqmule.usc.edu/) pipeline as our primary variant calling pipeline. In SeqMule pipeline, BWA (Li and Durbin, 2009) was used for sequence alignment and GATK (McKenna *et al.*, 2010) and SAMtools (Li *et al.*, 2009) were used to generate variant calls and to identify consensus calls for analysis. For further comparison between various variant-calling tools, such as SOAPsnp/SOAPindel, VarScan2 (Koboldt *et al.*, 2012), and SAMtools, we used variants from five candidate genes from five exome-sequenced patients as benchmarking data and generated variants from SOAPsnp/SOAPindel, VarScan2, GATK and SAMtools, respectively.

On the other hand, we decided to use VarScan2 as our primary variant calling tools for targeted region sequencing data analysis given sufficient sequencing depth. Our goal is to obtain the most somatic and germline variants calling from these cancer tissues because we are not only interested in the overlapped variants from exome sequencing but also in additional variants that occurred in these genes. Therefore, for this deeply sequenced data on which most pipelines can generate reliable variant calls, we chose VarScan2 as it is widely used in cancer mutation identification and that it is more sensitive for somatic mutations.

Mutation annotation

We used wANNOVAR web server at University of Southern California (http://wannovar.usc.edu/) to annotate all the mutations (Chang and Wang, 2012; Wang *et al.*,

2010). wANNOVAR server provides an easy-to-use interface to help researchers filter down variants, and judge the deleteriousness of variants using multiple sources of information, such as SIFT (Kumar *et al.*, 2009), PolyPhen-2 (Adzhubei *et al.*, 2010), and LRT (Chun and Fay, 2009) scores. Given the lack of paired normal tissues, we applied two filtering processes to sift for potential novel somatic mutations occurred in our patients. First, to filter for rare novel mutations and thus can potentially be somatic mutations, we discarded all mutations with Minor Allele Frequency (MAF) strictly greater than 0.001 from 1000 Genomes Project and NHLBI-ESP data on 6,500 exomes, and required the remaining mutations to be absent in dbSNP Build 138 and in 46 unrelated individuals from Complete Genomics, using wANNOVAR with corresponding filtering options. Second, given that our patients were all from Chinese population, which was less represented in these aforementioned databases, to filter for novel somatic mutations specific to Chinese population, we also obtained mutations from a recently published exome-sequencing project on around 1,000 Chinese people and checked the existence and allele frequencies of all remaining mutations in the five frequently mutated genes and in TRAF7.

Sanger validation

All candidate point mutations identified from exome sequencing were chosen for Sanger validation. Genomic sequences around each candidate mutations were obtained from NCBI. Twelve pairs of primers were designed using primer 3 (v. 0.4.0) (http://bioinfo.ut.ee/primer3-0.4.0/).

Next, we subjected DNA samples of all five patients to PCR using these 12 pairs of primers to validate all candidate mutations. The PCR mixture contained 5 μl of 10X AccuPrime™ PCR Buffer, 0.3 μl of AccuPrime™ Taq High Fidelit, 2 μl of 10 μM forward primer, 2 μl of 10 μM reverse primer, and 10 ng of template DNA, resulting in final volume of 50 μl. All PCR were performed under following conditions: 2 minutes at 94°C; 20 cycles of 20 seconds at 94°C and 30 seconds at 60°C (−0.5°C each cycle) and 1 minute at 70°C; 20 cycles of 20 seconds at 94°C and 30 seconds at 50°C and 1 minute at 70°C; and 7 minutes at 70°C.

After amplification of DNA sequence, we performed Sanger Sequencing of all 5 samples. The sequencing traces were manually visualized in Codon Code Aligner software (http://www.codoncode.com/aligner/) to confirm the presence of candidate mutations.

Results

Whole-exome sequencing identifies candidate genes

To survey the mutational landscape of malignant meningiomas, we performed whole-exome sequencing on five meningiomas on the Illumina HiSeq2000 platform using Agilent SureSelect Human All Exon kits. After removing low-quality reads, we obtained on average 45 million paired-end 90 bp reads per sample. Alignment statistics showed mean depth coverage of 54X, and 93.4% of bases were covered by at least 10X. In total, these five samples carried ~62,000 unique variants as our candidate variants for functional annotation and analysis.

In order to narrow the list of potential disease-predisposing variants and genes, we applied the 'variants reduction' pipeline implemented in the wANNOVAR server and identified a small subset of candidate variants and genes that are more likely to be deleterious (Chang and Wang, 2012). We focused on the list of non-synonymous SNVs, splice variants, and indels in exonic regions, given that they might be more interpretable and perhaps more likely to be disease associated. From this pipeline, we prioritized on average 170 novel protein-altering variants per sample (Table 2, Figure 1). Among all 799 genes with these novel mutations, 39 of them were shared by at least two patients and 4 of them were shared by at least three patients.

In order to further narrow down the list of candidate genes, we used DAVID web server and examined the function of all 799 genes (Huang da *et al.*, 2009). Manual examination of brain cancer-related keywords resulted in a ranked list of cancer genes, including *NF2*, *MN1*, *ARID1B*, *SEMA4D*, and *MUC2*. Sanger sequencing validated the presence of all point mutations (Figure 2). Due to the very small sample size, we decided to zoom into these five candidate genes and assess their likelihood of being associated with malignant meningiomas.

Among these five candidate genes, *NF2* encodes a membrane-cytoskeleton scaffolding protein, predominantly found in nervous tissue and is mainly located in adherens junctions. Previous studies showed that *NF2* was disrupted in approximately half of all meningiomas (Choy *et al.*, 2011). Moreover, in Cancer Gene Census (Futreal *et al.*, 2004), both germline mutations and somatic mutations in *NF2* are reported to be associated with meningiomas. *MN1* gene was originally cloned from the breakpoint of t(4;22)(p16;q11) in human meningioma and was suspected to contribute to its pathogenesis (Lekanne Deprez *et al.*, 1995). Moreover, as mentioned above, the distinctive repression of *MN1* was established in our pilot studies from analysis on transcriptome copy number alterations of malignant meningiomas (Chang *et al.*, 2013). *MUC2* encodes a transcription coregulator that plays an important role in diagnostic and prognostic prediction and in carcinogenesis and tumor invasion. It has been tested for association with the recurrence and outcome of pancreatic carcinoma and colorectal carcinoma (Hamada *et al.*, 2005; Imai *et al.*, 2013). *ARID1* family genes encode integral components of the SWI/SNF neural progenitor-specific chromatin-remodeling BAF complex that potentially plays a tumor-suppressor role in several cancers (Wilson and Roberts, 2011). For example, tumor-specific deletions encompassing *ARID1B* have been reported in CNS tumors (Ichimura *et al.*, 2006) and somatic mutations in *ARID1B* have also been reported to be associated with childhood cancer neuroblastoma (Sausen *et al.*, 2013). *SEMA4D* encodes axon guidance protein that regulates the functional activity of axons in the nervous system, whose physiological roles have been extensively explored in many facets of tumor progression, such as tumor angiogenesis, regulation of tumor-associated macrophages, and control of invasive growth (Ch'ng and Kumanogoh, 2010). Note that interference with *SEMA4D*-mediated pathways could be a viable adjunct to anti-VEGF therapy (Zhou *et al.*, 2012).

In addition, we found *TRAF7* mutation (c.1922G>C:p.R641P) in Patient 1, which is consistent with previous findings supporting the association between mutations in *TRAF* and benign/atypical meningiomas (Clark *et al.*, 2013). However, since when we ordered the

targeted region sequencing capture array, we did not include this gene into consideration, we did not present result of further validation in an additional cohort of patients. Nevertheless, *TRAF7* may still be valuable in further replication studies in the future.

Validation of candidate genes in an additional cohort

Despite the rarity of this cancer subtype, we obtained DNA samples from four additional patients with malignant meningiomas, and performed custom-designed target sequencing of five candidate genes using NimbleGen capture arrays, aiming to identify additional mutations from these candidate genes. Since the targeted region sequencing captures a smaller region compared to WGS or WES, we were able to obtain sequencing data with higher depth coverage on the targeted regions: on average, the read depth is 455X and 100% of targeted regions are covered.

We observed two recurrent mutations (c.979G> A:p.A327T and c.2138G>A:p.R713K) on gene *SEMA4D* that occurred in Patients 6 and 7. Note that since these two mutations were not novel (MAFs are 0.16 and 0.26 in the 1000 Genomes Project, respectively), they did not appear in the final set of novel variants obtained using variant reduction pipeline that filters for novel variants in WES data analysis stage. We also identified additional novel protein-altering mutations in *NF2*, *MN1*, and *MUC2* (Table 3, Table 4). We did not detect any additional protein-altering mutations on *ARID1B* in the validation stage. On the other hand, we were able to detect 104 additional mutations in *MUC2* gene, suggesting potential problems with alignment or variant calling (as many paralogs of mucins are present in human genome). We identified one protein-altering mutation (c.632C>T:p.A221V) in *NF2* across all four patients; notably, another missense mutation at the same position (c.632C>A) was reported in COSMIC database as a confirmed somatic mutation shared by two patients with meningiomas (Forbes *et al.*, 2011). In summary, the validation sample set provided additional evidence that *NF2* and *MN1* might be involved in meningioma pathogenesis.

Discussion

In this study, we applied WES on five malignant meningiomas and identified several candidate genes that may be associated with malignant transformation of meningiomas. To further validate the functionality of these genes on meningiomas, we deep-sequenced them on an additional set of four malignant meningiomas and found two recurrent mutations in gene *SEMA4D*, two novel mutations in *MN1*, and one missense mutation in *NF2*, which is also a confirmed meningioma somatic mutation in the COSMIC database. To our knowledge, our study is the first sequencing study focused on malignant meningiomas.

We acknowledge several challenges and limitations of the current study. First, unlike several large-scale genomic studies profiling somatic variations on meningiomas, our study lacks blood DNA from control subjects, thus the ability to distinguish somatic mutations from germline mutations is limited (Brastianos *et al.*, 2013; Clark *et al.*, 2013; Reuss *et al.*, 2013). Therefore, we relied on filtering procedure on multiple databases to remove known germline variants, but the ‘somatic’ mutation calls may still contain genuine germline mutations that were private to these Chinese patients, as these databases were not specific to Chinese population. We therefore calculated the mutation frequencies of all novel mutations detected

in the five frequently mutated genes as well as in *TRAF7* (which was also discussed in the Results section), using WES data from around 1,000 Chinese individuals (Tang *et al.*, 2014). We found that only one of the fourteen mutations (c.2138G>A:p.R713K in *SEMA4D*) has occurred with mutation frequency of 19% in this Chinese cohort, which means that except for c.2138G>A, all other mutations are unlikely to be germline variants specifically in Chinese population. Even with this analysis, we were still not able to validate their somatic status, given the absence of paired normal samples. Such lack of confirmed somatic mutations therefore reduced the power to detect cancer driver genes. For example, we could not use computational tools, such as MutSigCV used in Brastianos *et al.*'s study, to predict cancer driver genes, due to the unavailability of information of normal tissue samples and limited sample size. Second, the brain tissue is difficult to obtain and the availability of high-quality DNA samples from Formalin-Fixed Paraffin Embedded (FFPE) tissues were limited, even though additional patient samples were available. This issue is not uncommon for WES studies using FFPE tumor tissues, but previous studies demonstrated that 84.9% of the FFPE SNVs were common to fresh frozen tumor samples (Menon *et al.*, 2012), suggesting that FFPE may still be used for studying mutational spectrum of tumor samples. The same problem on FFPE also emerged during Sanger Sequencing validation stage. We even noticed that some validated variants actually came from low quality calls (quality <=30), which affected the reliability of the validation result. Third, malignant meningioma is an extremely rare disease with approximate incidence of 1.2 per million individuals according to the most recent Central Brain Tumor Registry of the United States report, released in 2010 (Ostrom *et al.*, 2013). This rarity makes tumor samples extremely hard to find and challenges data analysis. For example, we observed one *NF2* mutation in five exome sequenced patients and one *NF2* in four targeted region sequenced patients, but this gene failed to achieve statistical significance since the sample size was too limited. Such difficulty was tackled by two other related studies by analyzing pooled meningiomas samples with different subtypes (Brastianos *et al.*, 2013) or focusing on the more common benign subtype (Clark *et al.*, 2013). However, we would not be able to apply these two approaches, because our focus was on the malignant meningiomas. The complexity of this disease, especially in sporadic cases, further complicates our study, since multiple genes rather than one single gene may be drivers for cancer progression.

Nevertheless, our primary goal was to do an exploratory analysis using valuable data from these nine patients with malignant meningiomas, hoping to get a big picture of this disease on a whole-genome scale and find some interesting points to pin down in further research. Even though similar studies have been done on benign meningiomas (Clark *et al.*, 2013), it was the first time that five malignant meningiomas were sequenced on whole exome and that additional malignant meningiomas were sequenced by capture region sequencing. Therefore, data from our study provides valuable information on the genomic landscape of this rare but highly progressive disease. Moreover, to ensure the reliability of variant calls from whole exome sequencing results, we did a comparative approach by using two independent variant calling tools [GATK (McKenna *et al.*, 2010) and SAMtools (Li *et al.*, 2009), implemented by SeqMule pipeline] and by using the consensus variants from both variant calling tools as candidate variants for further validation, after observing

inconsistencies of variants generated from different tools, such as SOAP, GATK, VarScan2, and SAMtools.

In conclusion, our study represents an initial effort to define solely in malignant meningiomas the genomic spectrum of genetic alterations using NGS technology. In combination with various bioinformatics and biostatistics tools, we hope to understand this rare but deadly disease, to complement its current genomic profile and to help identify potential drug targets on these therapeutically challenging tumors.

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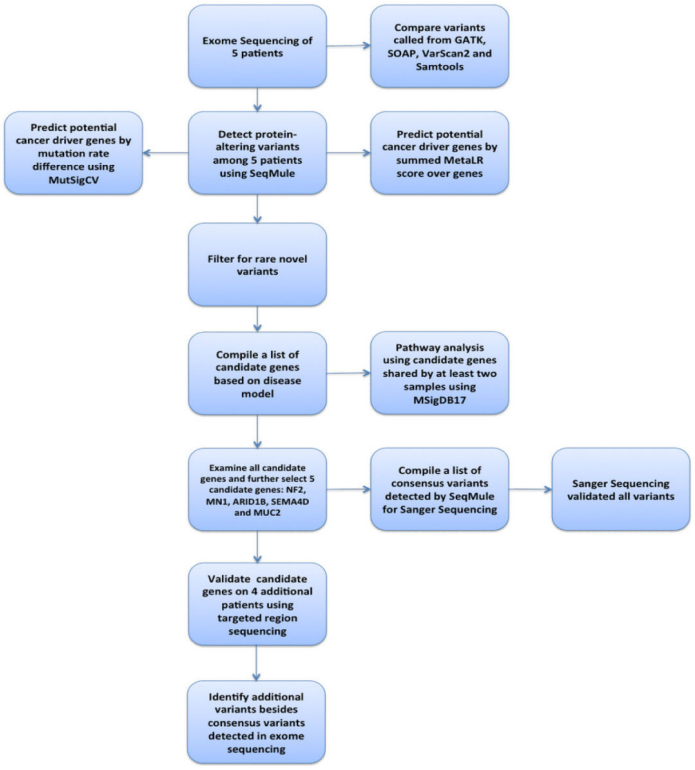

Figure 1.
Workflow of data analysis procedure in our study.

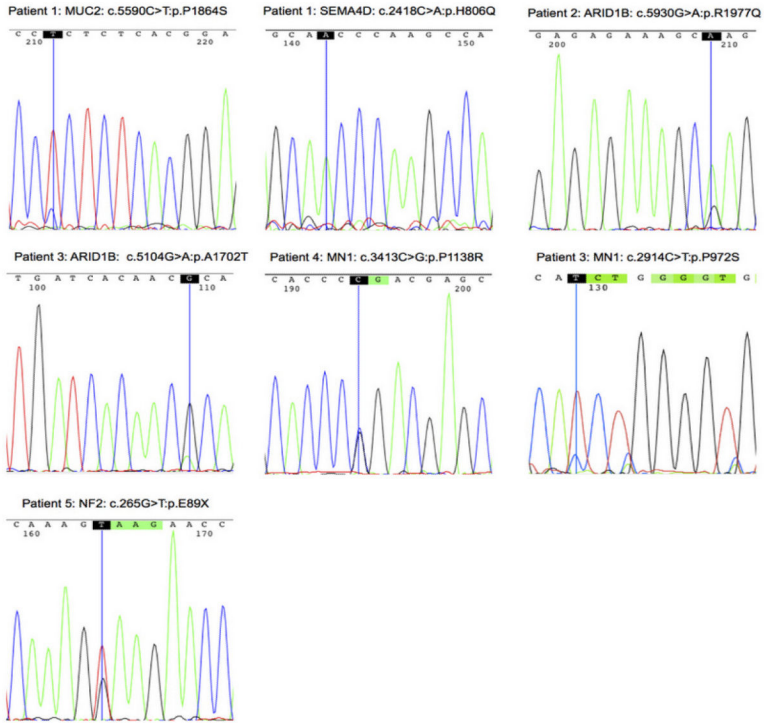

Figure 2.
Sanger Sequencing validation for mutations detected from exome sequencing.

Table 1

Description of Patients with Malignant (Grade III) Meningiomas.

ID	Gender	Age	Site	Status	Radio-therapy	Date of operation	Comments
1	F	28	Convexity (frontal lobe)	Primary	-	2003.09	-
2	M	66	Cranio-orbit	Recurrent	-	2005.01	Accepted surgery before half year due to meningioma (Grade II). Local recurrence occurred in November 2006. Metastasis to the lung.
3	M	30	Cerebellopontin angle	Primary	-	2006.03	Accepted radiation before 8 years due to nasopharyngeal carcinoma
4	F	49	Convexity (parietal lobe)	Primary	Yes	2010.06	-
5	F	58	Cerebellum	Primary	-	2010.06	-
6	F	56	Cranio-orbit	Primary	-	2003.07	Postoperative recurrence in 2007
7	M	26	Convexity (frontal and parietal lobe)	Primary	Yes	2008.07	-
8	F	11	Convexity (occipital lobe)	Primary	Yes	2009.1	-
9	M	45	Parasagittal	Recurrent	-	2010.11	Accepted operation in 2004 because of grade II~III meningioma

Table 2

Variants Filtering Pipeline for Five Exomes.

ID	1	2	3	4	5	Filtering Options
Variants	36252	35497	35574	33922	35019	No filtering
Step 1	7844	7553	7692	7403	7533	Identify missense, nonsense and splice variants and indels
Step 2	789	715	770	734	763	Remove variants in the 1000 Genomes Project (ALL) with MAF >0.001
Step 3	570	489	542	513	536	Remove variants in NHLBI-ESP 6500 exomes with MAF >0.001
Step 4	200	148	186	165	190	Remove variants in dbSNP138 (excluding clinically associated SNPs)
Step 5	199	145	184	161	189	Remove variants found in cg46 database
Step 6	188	141	176	158	182	Compile a list of candidate genes based on disease model
Candidate genes	MUC2, SEMA4D	ARID1B	ARID1B, MN1	ARID1B, MN2	NF2	Manually examining gene functions according to annotations in DAVID

Table 3

List of Novel Protein-altering Variants in Candidate Genes Compiled from Whole-exome Sequencing Data on Five Patients, Based on Two Independent Variant Calling Tools.

ID	Gene	Function	Type	Protein Effects	Zygosity	Read Depth
3	ARID1B	Non-synonymous	SNV	c.5104G>A:p.A1702T	Het	105
2	ARID1B	Non-synonymous	SNV	c.5930G>A:p.R1977Q	Hom	69
3	MN1	Non-synonymous	SNV	c.2914C>T:p.P972S	Hom	12
4	MN1	Non-synonymous	SNV	c.3413C>G:p.P1138R	Hom	14
1	MUC2	Non-synonymous	SNV	c.5590C>T:p.P1864S	Het	171
4	NF2	Frameshift	Deletion	c.1231delC:p.Q411fs	Het	17
5	NF2	Stopgain	SNV	c.265G>T:p.E89X	Het	53
1	SEMA4D	Non-synonymous	SNV	c.2418C>A:p.H806Q	Het	69

Table 4

List of Protein-altering Variants in ARID1B, MN1, NF2, and SEMA4D Identified in Four Additional Samples from Targeted Region Sequencing Data.

ID	Gene	Function	Type	Protein effects	Zygosity	Read Depth	Novelty
6	MN1	Non-frameshift	Insertion	c.1619_1620insGCA:p.540insQ	Het	68	Yes
7	MN1	Non-frameshift	Insertion	c.1598_1599insGCA:p.533insQ	Het	40	Yes
8	NF2	Non-synonymous	SNV	c.632C>T:p.A221V	Het	598	Yes
6	SEMA4D	Non-synonymous	SNV	c.979G>A:p.A327T	Het	264	No
7	SEMA4D	Non-synonymous	SNV	c.979G>A:p.A327T	Het	157	No
8	SEMA4D	Non-synonymous	SNV	c.2138G>A:p.R713K	Het	261	No