

AD _____

Award Number: W81XWH-12-1-0136

TITLE: Oncogene Discovery in Schwannomas

PRINCIPAL INVESTIGATOR: Peleg Horowitz

CONTRACTING ORGANIZATION: Dana-Farber Cancer Institute
Boston, MA 02215-5450

REPORT DATE: July 2013

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE July 2013		2. REPORT TYPE Annual Summary		3. DATES COVERED 15 April 2012 – 14 June 2013	
4. TITLE AND SUBTITLE Oncogene Discovery in Schwannomas				5a. CONTRACT NUMBER W81XWH-12-1-0136	
				5b. GRANT NUMBER W81XWH-12-1-0136	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Peleg Horowitz E-Mail: phorowitz@partners.org				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Dana-Farber Cancer Institute Boston, MA 02215-5450				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT About half of schwannomas are driven by loss of the NF2 tumor suppressor gene; however, a major barrier to more effective therapies is the lack of a comprehensive understanding of the genetic events outside of the NF2 gene in these tumors. In this project, we hypothesized that additional point mutations, copy-number changes, and fusion events between distant regions of the genome, drive the development of these tumors. To address this hypothesis, we have obtained schwannoma tissue and matched blood and begun examining the somatic genetic alterations through next-generation sequencing. To date, five pairs have undergone exome sequencing, while two additional samples are undergoing whole-genome sequencing. Many additional samples from new collaborators are scheduled for additional sequencing. Interestingly, among the five whole-exome sequenced schwannoma samples, the mutation rates are low, with three of the five samples harboring NF2 mutations. Additionally, we have identified a mutation in the proto-oncogene CBL in one sample and a genomic translocation disrupting RB1 in another sample, though both of these findings will require further validation. These findings begin to shed some light on the molecular complexity of schwannoma formation, and analysis of additional samples will greatly assist in our efforts.					
15. SUBJECT TERMS Schwannoma, neurofibromatosis, NF2, genetics, next-generation sequencing					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

TABLE OF CONTENTS

Face Page.....	1
SF298.....	2
Table of Contents	3
Introduction	4
Specific Aims	5
Progress Report (Body).....	5-8
Key Research Accomplishments.....	8
Reportable Outcomes	8-9
Conclusions	9
References.....	10

Progress Report

Department of Defense NFRP Post-Doctoral Fellow Grant NF110103:

Oncogene Discovery in Schwannomas

Peleg M. Horowitz, MD/PhD

Introduction

The bilateral appearance of vestibular schwannomas (acoustic neuromas) is the defining feature of the Neurofibromatosis type 2 syndrome.¹ Most schwannomas have benign histology, but their clinical impact on NF2 patients is anything but benign, causing progression from tinnitus, imbalance, and hearing loss to facial nerve dysfunction, brainstem compression, and early death. Nearly half of patients presenting with unilateral schwannomas will go on to develop bilateral tumors within 20 years of diagnosis, and this rate increases to over 80% if the first tumor is diagnosed in childhood.² The proximity of these tumors to facial and acoustic nerves and brainstem makes their treatment quite challenging. Although surgery can be beneficial, particularly in cases of brainstem compression, it carries significant risk of hearing loss and facial dysfunction, and often an incomplete resection is required to prevent injury to critical structures.³ Radiotherapy is similarly plagued by delayed injury to the cranial nerves and failure to control tumor growth, so that resection is ultimately necessary.⁴ Few adjuvant therapies have been developed, and these are variably effective in durable tumor control. Consequently, recurrence and progression of schwannomas in NF2 after surgery and radiotherapy are common.

The development of new treatments for schwannomas is hampered by our limited understanding of their evolution. Early investigations into the causes of neurofibromatosis 2 showed linkage to the merlin / schwannomin gene on chromosome 22.⁵⁻⁸ Proposed mechanisms for the activity of the merlin/schwannomin tumor suppressor gene include its association with the p21 kinase pathway⁹, mTORC1¹⁰, and e3 ubiquitin ligase¹¹.

While the role of the schwannomin/merlin gene as a tumor suppressor in both sporadic and neurofibromatosis cases is well described, additional *de novo* somatic genetic events driving schwannoma tumorigenesis outside the *NF2* gene are clearly present and poorly understood. Cytogenetic analysis of schwannomas using Comparative Genomic Hybridization (CGH) has revealed additional regions of recurrent DNA aberration outside chromosome 22.¹²⁻¹⁴ Loss of 22q (containing *NF2*) occurs reproducibly in 24-29% of tumors, but nearly half of the cytogenetic anomalies in these tumors are located elsewhere, most frequently in chromosomes 19 (35%), 16 (30%) and 9q (10%). Interestingly, alterations in other genes on chromosome 22 (*SMARCB1*, *GSTT1* and *CABIN1*) have also been associated with schwannoma development in Neurofibromatosis and familial schwannomatosis.¹⁵⁻¹⁷

It is clear that schwannomas undergo many genetic events outside the *NF2* gene that have the potential to be involved in tumor formation. However, to date none of these events have been shown to be driver events or validated as oncogenes or tumor suppressors in model systems.

A major barrier to more effective therapies is the lack of a comprehensive understanding of the genetic events outside of the NF2 gene in these tumors. In this project, we hypothesized that additional somatic genetic events, consisting of point mutations, copy-number changes, and fusion events between distant regions of the genome, drive the development of these tumors. Understanding the complete landscape of these events will reveal additional putative oncogenes and tumor suppressors whose aberrant activity is responsible for tumor formation.

To address this hypothesis, two specific aims have been proposed:

Aim 1: Comprehensively characterize schwannoma genomes for all possible point mutations, copy-number changes, and fusion events that may contribute to schwannoma oncogenesis by whole genome sequencing of 20 schwannomas and normal tissue from the same patients.

Aim 2: Validate and gauge the frequency of mutations identified in Aim 1 by performing focused assays for these events across a panel of 200 additional schwannomas.

Progress Report (Body)

Since the start of the project, we have made significant advances, particularly in (1) the establishment of international collaborations to obtain tissue, (2) identification and extraction of schwannoma and paired normal DNA from BWH/DFCI Brain Tumor Bank, and (3) whole-exome sequencing of samples, despite several obstacles we have encountered and addressed.

Tissue Acquisition

After querying our institutional tissue bank (the Dana-Farber Cancer Institute / Brigham and Women's Hospital Brain Tumor Repository), we discovered that while many schwannomas were archived in the bank, there were a number of factors limiting the number of samples amenable to sequencing for our study.

First, a number of the older samples (pre-2007), while in excellent condition, lacked a paired blood (normal) sample for comparison, necessary to allow identification of mutations specific to the tumors (somatic mutations, as opposed to germline variations). Prior to approximately six years ago, the importance of collecting germline DNA samples for comparison was poorly understood, and at our institution (as well as many others), only the tumor specimens were collected. Unfortunately, our IRB does not provide for re-contacting patients to obtain additional samples. These samples lacking normal DNA, while not usable for Aim 1 (discovery-phase genomic sequencing) will be of utility in Aim 2 (validation focused sequencing).

Second, the quantity of available tissue per tumor was in some cases a limiting factor. Schwannomas range greatly in size (from small sub-centimeter intracranial tumors to large brainstem-compressing lesions) as well as in consistency (from cellular and solid to cystic and liquid). Therefore, of the twenty tumors identified for discovery sequencing in Aim 1, only two had sufficient DNA yield (> 6 micrograms) for 30x coverage whole-genome sequencing as initially proposed. Of the remaining 18 tumor-normal pairs, however, 12 samples had sufficient DNA yield for whole-exome sequencing, a method of genomic analysis that involves an additional DNA enrichment and amplification step (hybrid capture selection). While the entire genome is not covered by this method, over 90% of the exons (protein-coding regions) are covered; this allows detection of mutations and insertion-deletions, as well as inference of copy number state, but limits the detection of intra- and inter-chromosomal rearrangements.

Given these limitations in tissue for the proposed project, we initiated an international search for collaborations. Our resulting collaboration with the Kolling Institute of Medical Research Cancer Genetics Unit at the University of Sydney Neuroendocrine Surgical Unit is particularly exciting because the Kolling Institute has approximately 100 banked frozen schwannoma samples, many of these with paired normal blood samples. Approximately one third of these tumor samples come from intracranial schwannomas, the remainder are from peripheral nerve tumors. The Kolling Institute laboratories also have expertise in RNA analysis, and our Australian collaborators are interested in sharing these samples for combined DNA analysis (under this DoD NFRP project) and RNA analysis (to be performed at the University of Sydney). This collaboration will greatly increase the number of samples available to meet our objectives of sequencing in Aims 1 and 2. At this point, we have obtained final Ethics Committee approval from the Kolling Institute (and have already obtained Dana-Farber Cancer Institute IRB approval) to proceed with the tissue transfer. We are also initiating collaboration with the University of Toronto, though this is in the early stages.

Despite the obstacles in acquiring large numbers of schwannomas with paired blood and sufficient DNA, we have made exciting progress in analyzing the samples that are available to us at present. The status of these samples in our analysis pipeline is detailed in the flowchart below (Figure 1).

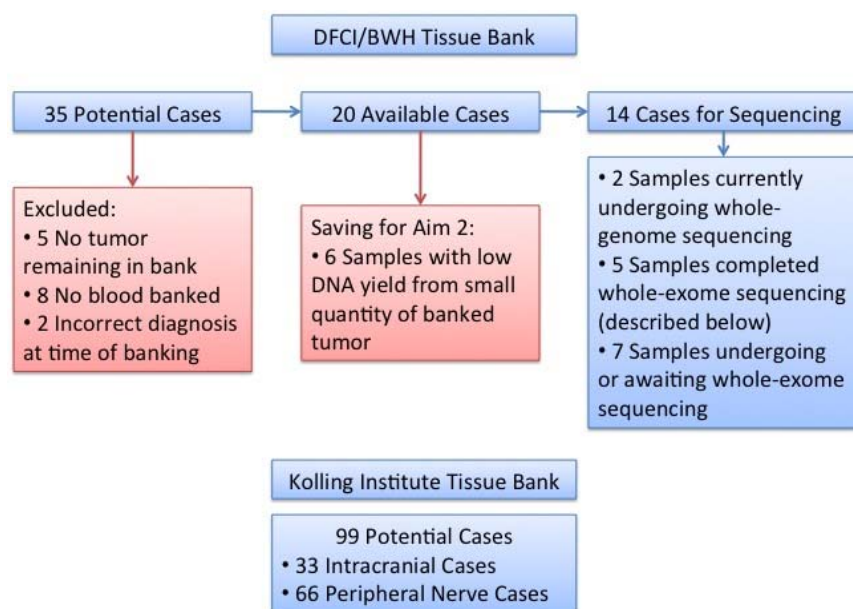


Figure 1. Obstacles to obtaining, and progress in analyzing, the schwannoma tumor-normal sample pairs to date.

Sequencing Results

Five schwannoma tumor-normal pairs have completed whole-exome sequencing at the Dana-Farber Cancer Institute Center for Cancer Genome Discovery and have been fully analyzed for mutations, insertion-deletions, copy number alterations, and limited analysis for translocation rearrangements. An overview of the findings in these five tumors is shown below in Figure 2.

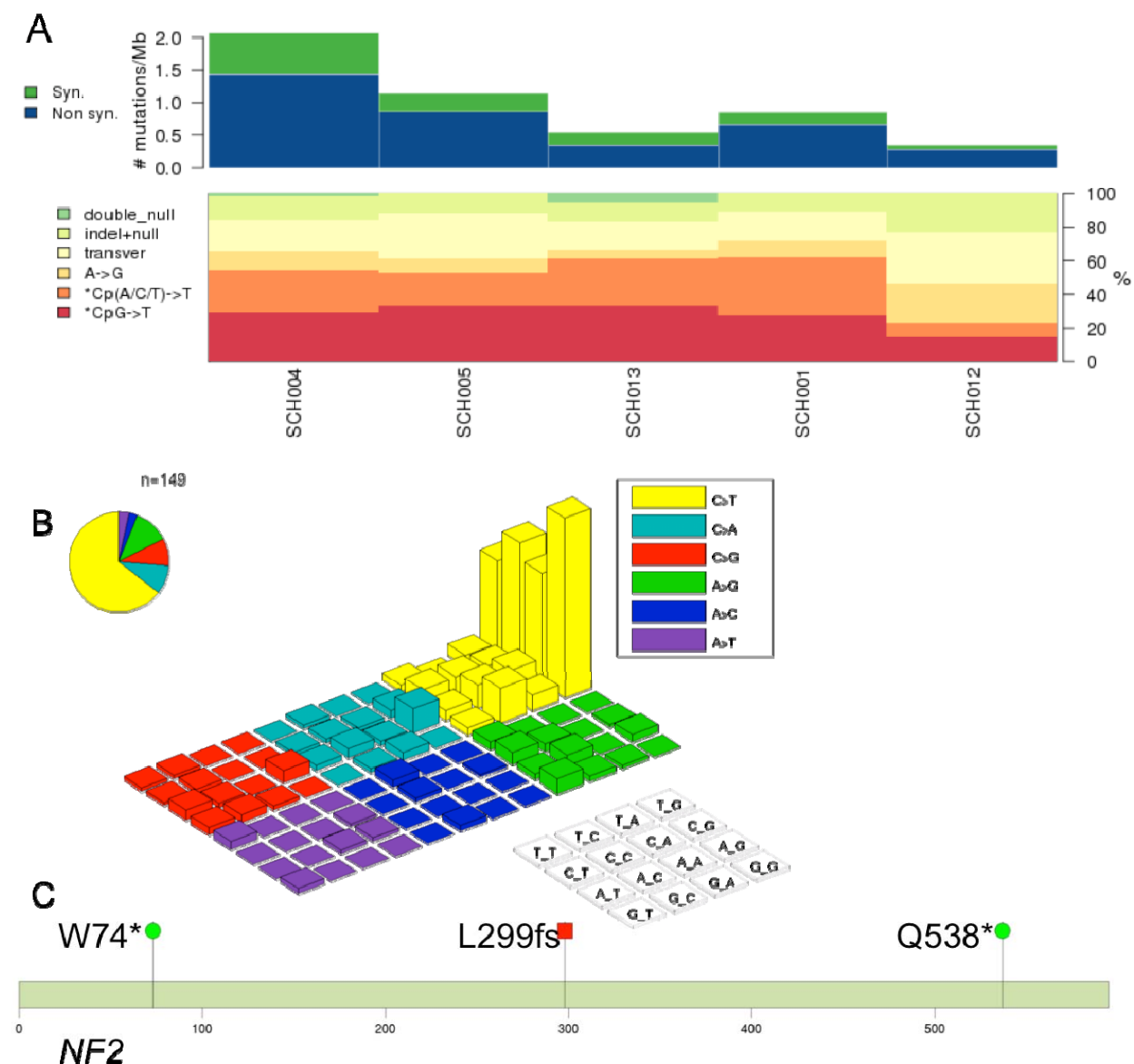


Figure 2. Overview of mutations in five whole-exome sequenced schwannomas. **A.** Top: The rate of non-silent (dark blue) mutations in the five schwannomas ranges from 0.3-1.4 mutations per Mb, relatively low compared to all sequenced cancers but similar to recently profiled meningiomas¹⁸. Bottom: the spectrum of all mutation types shows an even mix of missense (red, orange) and deleterious (nonsense, splice site, and frame-shift; yellow and green) mutations. **B.** The single-nucleotide mutations in schwannomas are primarily of the spontaneous deamination type (yellow), suggesting that these tumor-specific mutations are a result of a spontaneous, naturally occurring process rather than a known carcinogen. A similar pattern is also seen in meningioma¹⁸. **C.** Three of the five samples had mutations in the *NF2* gene; all three were disruptive (two non-sense (green circles) and one frame-shift deletion (red square)).

In addition to the three *NF2* mutations shown above, a point mutation in the proto-oncogene *CBL* (Cas-Br-M (murine) ecotropic retroviral transforming sequence) was identified in one sample,

and this mutation matches one previously demonstrated in the Catalogue of Somatic Mutations in Cancer (COSMIC). Two similar *CBL* mutations were recently described in meningiomas¹⁸.

As described above, exome sequencing, while covering >90% of the protein-coding portions of the genome, only provides a limited glimpse at the genomic rearrangements that often take place in non-coding regions. However, analysis for rearrangements in these tumors uncovered two such events, one is shown below (Fig. 3).

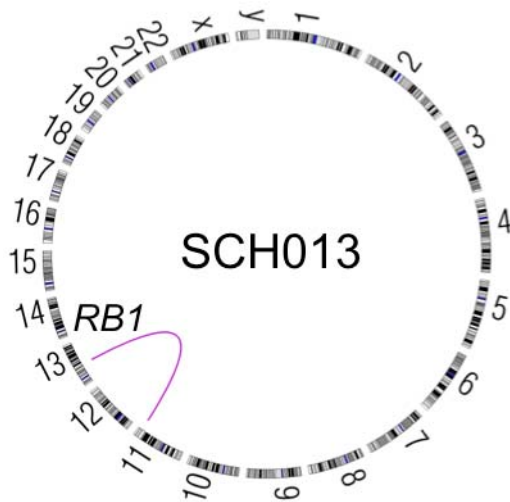


Figure 3. Analysis of genomic rearrangements in the five exome-sequenced schwannoma samples using the dRanger and BreakPointer algorithms identified this interchromosomal translocation between chromosomes 11 and 13 in sample SCH013. The rearrangement causes disruption of the well-known tumor suppressor gene *RB1* on chr13, which is fused to the *FEZ1* gene on chr11. Loss of *RB1* is a driver of several other tumor types¹⁹, but genomic loss of this gene in schwannomas has not been described.

Key Research Accomplishments

- Identification of 35 potential schwannoma cases in the DFCI/BWH tumor bank, of which two are currently undergoing whole-genome sequencing, five have completed whole-exome sequencing and analysis, and seven more are undergoing whole-exome sequencing
- Initiating an international collaboration with the Kolling Institute and University of Sydney, Australia to obtain additional 99 schwannoma samples with paired normal blood as well as undertake RNA analyses of these tumors
- Among the five whole-exome sequenced schwannoma samples, the mutation rates are low, with three of the five samples harboring *NF2* mutations
- Identification of a mutation in the proto-oncogene *CBL* in one sample and a genomic translocation disrupting *RB1* in another sample, though both of these findings will require further validation in additional samples

Reportable Outcomes

Presentations

These findings have been presented in their preliminary format in a recent poster at the BWH Neurosurgery Research Retreat which included a report on the progress of this project, among others:

Horowitz P, Brastianos P, Santagata S, Jones R, McKenna A, Carter S, Palescandolo E, Van Hummelen P, Ducar M, MacConaill L, Stemmer-Rachmamimov A, Louis D, Ashley D, Hahn W, Ligon K, Beroukhirn R, and Dunn I. Genomics of Skull Base Tumors. Poster Presentation, Brigham and Women's Hospital Neurosurgery Research Retreat, 2013 (Boston, MA).

Once additional sequence data become available, I plan to present the data at local and national conferences as well as submit them for publication in a major peer-reviewed journal.

Peer-reviewed Publications

Additionally, we recently published a paper¹⁸ involving use and development of many of the algorithms described above, which will be used in continued analysis of schwannomas. The work in the publication relates to meningiomas, which share many features with schwannomas, including their association with Neurofibromatosis type 2:

Brastianos PK*, Horowitz PM*, Santagata S, Jones RT, McKenna A, Getz G, Ligon KL, Palescandolo E, Van Hummelen P, Ducar MD, Raza A, Sunkavalli A, MacConaill LE, Stemmer-Rachmamimov AO, Louis DN, Hahn WC**, Dunn IF**, Beroukhirn R**. (2013) Genomic sequencing of meningiomas reveals oncogenic SMO and AKT1 mutations. *Nature Genetics* **45**(3):285-9. PMID: 23334667.

As there is significant relevance to Neurofibromatosis and schwannoma with the above paper, the DOD NFRP was acknowledged as a funding source in this manuscript. I also authored an invited review article on "Genomic Characterization of Meningiomas", in press at the *European Association of NeuroOncology Magazine*, for which the DOD NFRP is acknowledged.

Grants

Since acceptance of the DOD NRFP grant, I have applied for and successfully obtained a research grant from the Brain Science Foundation for the purposes of continuing to study the genomic alterations in schwannomas. As the DOD NFRP covers primarily salary and stipend, this new grant from the Brain Science Foundation, which spans 2 years and carries a \$140,000 total budget, will cover almost entirely sequencing costs and other expenses directly related to the research. The grant will allow for continuity of the research after conclusion of the DOD NFRP this July, as the BSF grant terminates in October of 2014.

Conclusions

Though several obstacles were encountered in the tissue acquisition phase of the project, we were successfully able to both obtain sufficient DNA for sequencing from 14 paired tumor-normal schwannoma cases, as well as strike up a promising collaborative relationship with the Kolling Institute in Australia to obtain many additional samples. Preliminary analysis of the five sample pairs that have completed whole-exome sequencing demonstrates that, in addition to three mutations in *NF2*, these tumors harbor additional interesting genetic abnormalities affecting oncogenes and tumor suppressors. Analysis of these new findings and discovery of further novel events in additional tumors will lead to a better understanding of schwannomagenesis and hopefully provide targets for future therapeutics.

References

1. Evans, D.G. Neurofibromatosis type 2 (NF2): a clinical and molecular review. *Orphanet J Rare Dis* **4**, 16 (2009).
2. Evans, D.G. *et al.* What are the implications in individuals with unilateral vestibular schwannoma and other neurogenic tumors? *J Neurosurg* **108**, 92-6 (2008).
3. Charalampakis, S., Koutsimpelas, D., Gouveris, H. & Mann, W. Post-operative complications after removal of sporadic vestibular schwannoma via retrosigmoid-suboccipital approach: current diagnosis and management. *Eur Arch Otorhinolaryngol* **268**, 653-60 (2011).
4. Yeung, A.H. *et al.* Radiobiology of vestibular schwannomas: mechanisms of radioresistance and potential targets for therapeutic sensitization. *Neurosurg Focus* **27**, E2 (2009).
5. Dunham, I. *et al.* The DNA sequence of human chromosome 22. *Nature* **402**, 489-95 (1999).
6. Rouleau, G.A. *et al.* Alteration in a new gene encoding a putative membrane-organizing protein causes neuro-fibromatosis type 2. *Nature* **363**, 515-21 (1993).
7. Rouleau, G.A. *et al.* Genetic linkage of bilateral acoustic neurofibromatosis to a DNA marker on chromosome 22. *Nature* **329**, 246-8 (1987).
8. Trofatter, J.A. *et al.* A novel moesin-, ezrin-, radixin-like gene is a candidate for the neurofibromatosis 2 tumor suppressor. *Cell* **72**, 791-800 (1993).
9. Chow, H.Y., Stepanova, D., Koch, J. & Chernoff, J. p21-Activated kinases are required for transformation in a cell-based model of neurofibromatosis type 2. *PLoS One* **5**, e13791 (2010).
10. James, M.F. *et al.* NF2/merlin is a novel negative regulator of mTOR complex 1, and activation of mTORC1 is associated with meningioma and schwannoma growth. *Mol Cell Biol* **29**, 4250-61 (2009).
11. Wei, D. & Sun, Y. Small RING finger proteins RBX1 and RBX2 of SCF E3 ubiquitin ligases: the role in cancer and as cancer targets. *Genes Cancer* **1**, 700-707 (2010).
12. Ikeda, T., Hashimoto, S., Fukushima, S., Ohmori, H. & Horii, A. Comparative genomic hybridization and mutation analyses of sporadic schwannomas. *J Neurooncol* **72**, 225-30 (2005).
13. Koutsimpelas, D., Felmeden, U., Mann, W.J. & Brieger, J. Analysis of cytogenetic aberrations in sporadic vestibular schwannoma by comparative genomic hybridization. *J Neurooncol* **103**, 437-43 (2011).
14. Warren, C. *et al.* Identification of recurrent regions of chromosome loss and gain in vestibular schwannomas using comparative genomic hybridisation. *J Med Genet* **40**, 802-6 (2003).
15. Bacci, C. *et al.* Schwannomatosis associated with multiple meningiomas due to a familial SMARCB1 mutation. *Neurogenetics* **11**, 73-80 (2010).
16. Buckley, P.G. *et al.* Identification of genetic aberrations on chromosome 22 outside the NF2 locus in schwannomatosis and neurofibromatosis type 2. *Hum Mutat* **26**, 540-9 (2005).
17. Rousseau, G., Noguchi, T., Bourdon, V., Sobol, H. & Olschwang, S. SMARCB1/INI1 germline mutations contribute to 10% of sporadic schwannomatosis. *BMC Neurol* **11**, 9 (2011).
18. Brastianos, P.K. *et al.* Genomic sequencing of meningiomas identifies oncogenic SMO and AKT1 mutations. *Nat Genet* **45**, 285-9 (2013).
19. Di Fiore, R., D'Anneo, A., Tesoriere, G. & Vento, R. RB1 in cancer: Different mechanisms of RB1 inactivation and alterations of pRb pathway in tumorigenesis. *J Cell Physiol* **228**, 1676-87 (2013).