Genetics of vestibular schwannoma

Genetic landscape of irradiated and radiation-naïve benign and malignant vestibular schwannoma

Aril Løge Håvik

Thesis for the degree of Philosophiae Doctor (PhD)
University of Bergen, Norway
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UNIVERSITY OF BERGEN

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Contents

1. Scientific environment

The present research project was performed during 2012 - 2021 through the Medical Student Research Program at The Faculty of Medicine (2012 – 2017) and through the Ph.D. program at the University of Bergen (2018 – 2021). During these years I have been affiliated at:

Department of Clinical Science
University of Bergen
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Department of Clinical Medicine
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Department of Medical Genetics
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Main supervisor:
Professor Per Morten Knappskog, MSc, PhD, Department of Clinical Science

Co-supervisors:
Professor Morten Lund-Johansen, MD, PhD, Department of Clinical Medicine
Ove Bruland, MSc, PhD, Department of Medical Genetics

2. Acknowledgements

When I first applied to medical school, back in 2010, it was with an aspiration to do research on the enigmatic function of the brain. I reckoned that that was the domain of the neurosurgeon, and hence looked up the name of a professor in neurosurgery at my faculty and sent him an e-mail explaining my interests. A few hours later, professor and neurosurgeon Morten Lund-Johansen put me in touch with professor and geneticist Per Morten Knappskog and geneticist Ove Bruland to include me in the vestibular schwannoma research group. I realized that this research was not about brain functioning. However, I have come to consider the genome as enigmatic and intriguing as the brain, and at least very fulfilling for me to study.

Per, my main supervisor, has always found time in his schedule to guide me through the hurdles of genetics. Through our years working together, we have shared numerous scientific discussions, coffee breaks, lengthy laughs, plans for the next skiing trip and where to catch the best cod. Together with Per, Morten started collecting tissue for the Bergen Neurosurgical Tissue Bank in 2001, enabling this project. Morten is an expert at getting things done and have also inspired me to do the same. He has shared his scientific and clinical wisdom with me over the years either at the office, at conferences, at the surgical theatre, in the mountains or at band rehearsal. Ove is a dedicated scientist with an impressive know-how when it comes to technical challenges both in the laboratory and in front of the computer. He has helped me out a lot when I get stuck and has been invaluable for the sequencing bioinformatics. The environment in our small research group has always been very friendly, inspiring and fruitful. Every time progress in my work stalled, I would call for a meeting to get a motivational boost. I am grateful and indebted to my supervisor team for everything you have taught me and for realizing this work.

Thanks to all my colleagues at The Department of Medical Genetics for providing a friendly atmosphere, expertise and the infrastructure needed to conduct this research. A special thanks to technicians Jorunn Skeie Bringsli for guiding me through my first nervous steps at the lab and Guri Matre for being my mentor and companion at the lab. Guri deserves extra credit for her excellence in laboratory techniques, her

efficiency, positive attitude and for keeping all the samples and experiments in order. Thanks also to Atle Brendehaug, Sigrid Erdal, Rita Holdhus, Stefan Johansson, Hilde E. Rusaas and Tomasz Stokowy for invaluable help along the way.

I am grateful to my colleagues at The Department of Neurogsurgery for both their contributions to this project and for providing a great environment for professional and personal development. A special thanks to Erling Myrseth, Terje Sundstrøm and Morten for operating VS patients and providing tissue. Thanks to Rupavathana Mahesparan, Head of the department, for trusting me with my first job as a doctor, providing a valuable clinical view of my research. Monica Finnkirk and Linda Karin Fauske at the National treatment center for vestibular schwannoma are credited for their contribution in managing the patient logistics.

Mads Aarhus paved the way for me in this research group, completing his thesis a year before I started. I am grateful for his guidance to get me started, for his contributions in formulating hypothesis and for his contribution to several of my papers. Thanks also to my other coauthors for your contribution, Hrvoje Miletic, Karl-Henning Kalland, Dhanushan Dhayalan, Lars Poulsgaard, David Scheie and Kåre Fugleholm. I am indebted to the Medical Student Research program for providing me with the opportunity to start doing research early in my career. None of this would have been possible without the contributions from all the patients, and for that I am grateful. The same could be said about my fellow PhD students, Eigir, Anny, Dhanushan, Tor-Arne, Dinka, Amund and Yasmeen, whom I have shared the struggles and joy of science with through the years.

Most importantly, a big thank you to my best friend, colleague and wife, Hilde-Kristin Hovde. You have inspired me both in life and work. The arrival of our daughter Eva did probably not accelerate my progress with this thesis, but it certainly made me a happier man. My mom (Tove Løge) and dad (Hans Alf Håvik) also deserves special credit in supporting and inspire me throughout my life.

Aril Løge Håvik
Molde, June 2021

3. Abbreviations

bp	Base pair of DNA
CN8	8th cranial nerve, the vestibulocochlear nerve
CNA	Copy number aberration
CNN-ROH	Copy number neutral run of homozygosity
CTLP	Chromothripsis-like pattern
DNA	Deoxyribonucleic acid
GKRS	Gamma knife radiosurgery
IGV	Integrative Genomics Viewer
Indel	Insertions and deletions of segments of DNA
IR	Ionizing radiation
Italic font	*Words in italic font denotes genes*
Kbp	Thousand (kilo) base pairs of DNA
Mbp	Million (mega) base pairs of DNA
MLPA	Multiplex ligation-dependent probe amplification
MPNST	Malignant peripheral nerve sheath tumor
NGS	Next-generation sequencing, aka second-generation sequencing and massively parallel sequencing
NF2	Neurofibromatosis type 2, an autosomal dominant multiple neoplasia syndrome
NF2	Neurofibromin 2 gene
PCR	Polymerase chain reaction
qRT-PCR	Quantitative reverse transcriptase real-time PCR
RIN	Radiation-induced neoplasia
RNA	Ribonucleic acid
RNAseq	Whole-transcriptome sequencing, utilizing NGS technology
RT-PCR	Reverse transcriptase PCR
SNV	Single nucleotide variant
SRS	Stereotactic radiosurgery
sVS	Sporadic vestibular schwannoma
VN-MPNST	Malignant peripheral nerve sheath tumor of the vestibulocochlear nerve, aka malignant vestibular schwannoma
VS	Vestibular schwannoma (aka acoustic neuroma)
WES	Whole-exome sequencing utilizing NGS technology
WGS	Whole-genome sequencing, utilizing NGS technology

4. Abstract

Background: Vestibular schwannoma (VS) is a benign intracranial neoplasm associated with reduced quality of life. Malignant peripheral nerve sheath tumor of the vestibular nerve (VN-MPNST) is the malignant counterpart, an exceedingly rare cancer associated with high mortality. The genetics underlying VS and its etiology is not well understood and the genome of irradiated VS and VN-MPNST has not been characterized. We addressed these shortcomings in this thesis.

Material and methods: Tumor specimens from the Bergen neurosurgical tissue bank were subjected to a combination of whole-exome sequencing (WES), whole-genome sequencing and microarray, MLPA, transcriptome sequencing, ViroChip and Sanger sequencing.

Results: A median of 14 (4-57) genes were mutated and a median of 0.17% of the autosome was affected by copy number aberrations (CNA) in VS. *NF2* mutation was observed in 89%. Tumors with wildtype *NF2* harbored mutations in genes linked to *NF2.* Novel genes and pathways identified in VS included *CDC27* (11%), *USP8* (7%) and axonal guidance pathway (54%). One clinically aggressive VS was identified and correlated with high mutational burden (231) and mutated *RAD54L.* Variant allele frequencies for both small mutations and CNAs indicated intratumoral heterogeneity. No plausible virus was associated with VS. We identified a premalignant VS characterized by large chromosomal aberrations and mutated *NF2*. Malignant transformation was accompanied by whole-genome doubling and mutations in *GNAQ, FOXO4* and *PDGFRB.* VN-MPNST is characterized by gross chromosomal aberrations and homozygous loss of *CDKN2A.* Previous treatment with GKRS in VS and VN-MPNST did not correlate with neither specific mutations nor genome wide signatures. COSMIC mutational signature 3 contributes to VN-MPNST while signature 6 contributes to a subset of VS.

Conclusion: VS is characterized by intratumoral genetic heterogeneity and relatively few mutations. We found recurrent mutations in *NF2* and the axonal guidance pathway in addition to novel genes in subsets. Mutated *RAD54L* might correlate with a hypermutator phenotype and worse clinical course. We identified *CDKN2A* as a likely tumor suppressor in both premalignant VS and VN-MPNST. Premalignant VS showed signs of chromosomal instability making it prone to malignant transformation. No biomarker of radioresistance or signature of exposure to ionizing radiation was identified in neither VS nor VN-MPNST. We found no evidence of a viral etiology in VS.

5. List of Publications

I. Havik AL, Bruland O, Myrseth E, Miletic H, Aarhus M, Knappskog PM, Lund-Johansen M (2017) Genetic landscape of sporadic vestibular schwannoma. Journal of neurosurgery: 1-12 doi:10.3171/2016.10.JNS161384

II. Havik AL, Bruland O, Aarhus M, Kalland KH, Stokowy T, Lund-Johansen M, Knappskog PM (2018) Screening for viral nucleic acids in vestibular schwannoma. Journal of neurovirology doi:10.1007/s13365-018-0669-6

III. Havik AL, Bruland O, Dhayalan D, Lund-Johansen M, Knappskog PM (2020) Gamma Knife Radiosurgery does not alter the copy number aberration profile in sporadic vestibular schwannoma. Journal of neuro-oncology doi:10.1007/s11060-020-03631-4

IV. Havik AL, Bruland O, Miletic H, Poulsgaard L, Scheie D, Fugleholm K, Lund-Johansen M, Knappskog PM (Under review) Genetic alterations associated with malignant transformation of a sporadic vestibular schwannoma.

6. Introduction

6.1 What is a vestibular schwannoma?

Vestibular schwannoma (VS) is a benign intracranial neoplasm originating from Schwann cells surrounding the 8th cranial nerve (CN8).[2] It is typically composed of well-differentiated cells, corresponding to WHO grade 1 tumor, and it never metastasize.[3] VS is sporadic in >95% of cases, the rest being associated with the autosomal dominant multiple neoplasia syndrome NF2, characterized by bilateral VS. VS makes up 8% of intracranial tumors, with an annual incidence rate of 20 per million, which results in around 100 new cases in Norway every year.[4,5] However, incidence seems to be on the rise, probably due to the increased availability of MRI in the community and increasing life-expectancy.[6] Median age and size at diagnosis is 55 years and 16 mm, respectively, and there is a negative correlation between age and size.[7] Typical presenting symptoms are unilateral hearing loss, tinnitus, vertigo and dizziness.[8] Adjacent cranial nerves (facial, trigeminal and lower cranial nerves) might also be affected. Larger tumors might compress the brain stem and cerebellum leading to dysmetria, ataxia, gait problems and hydrocephalus. If left untreated, the tumor might compress the cerebral aqueduct and cause obstructive hydrocephalus and lead to death. Except for the largest tumors, symptoms do not correlate strongly with tumor size. VS is in general an indolent tumor and only around 40% of extrameatal tumors

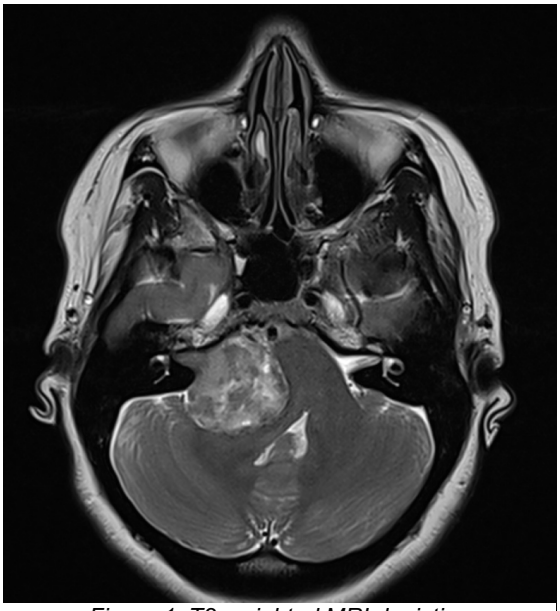

Figure 1. T2-weighted MRI depicting a large right-sided cystic vestibular schwannoma with brainstem compression and displacement of the 4th ventricle.

demonstrate growth 10 years after diagnosis, with some tumors even shrinking.[9] Immune cell infiltration and intratumoral bleeding, rather than neoplastic cell proliferation, correlate with tumor size and growth.[10-12] A high degree of intratumoral heterogeneity might be observed with tumor-associated macrophages making up as much as 70% of the cells in growing tumors.[13] The diagnosis is made based on typical appearance on MRI scan and, in the case of surgery, complemented by histopathological examination (*Fig.1*).[14] VS is typically composed of cellular areas of spindle cells (Antoni A) and hypocellular loose microcystic areas (Antoni B), although the molecular and cellular basis for these distinct morphological areas is not understood.[15] Although mortality rate is low in countries with readily access to neurosurgical treatment, there is significant morbidity leading to reduced quality of life [16-18]. Since the initial attempts to treat VS surgically in the 1890s, the goal of treatment has shifted from saving the patient's life to saving neurological function.[19] Today, four treatment options exist; 1) observation, 2) stereotactic radiosurgery (SRS), 3) fractionated radiotherapy and 4) microsurgery (*Fig.2*).[20] Although contemporary treatment of VS is successful in saving the patient's life, and even in preserving neurological function, the disease is still associated with significant morbidity.[21] Pharmacotherapy has so far been restricted to familial VS in the setting of NF2, including the

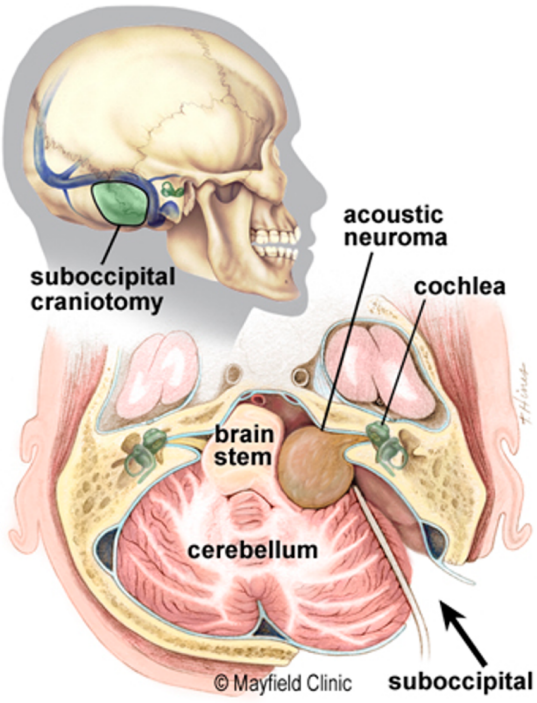

Figure 2. The suboccipital approach is used at our department where a small craniotomy is made behind the ear and the cerebellum is retracted to gain access to the tumor. Figure downloaded from https://www.mayfieldclinic.com/pe-acousticsurgery.htm

VEGF inhibitor bevacizumab.[22] Hence, new treatment options are needed for sporadic VS (sVS).

6.1.1 Risk factors and etiology of vestibular schwannoma

Established etiologies for VS include NF2, high doses of ionizing radiation from obsolete treatment of benign conditions in children (e.g., tinea capitis) and radiation doses experienced by atomic bomb survivors.[23-25] However, these would only account for a negligible portion of the VS patients encountered in an average clinic today. The peculiar fact that nearly all intracranial schwannomas originate from CN8 has led to different theories about its cause, including mobile phone use, noise exposure and viral infections. The role of mobile phone usage, a source of non-ionizing radiofrequency radiation, as a risk factor for developing VS has been extensively studied, with conflicting results.[26,27] In a large population-based cohort study in Denmark, Schüz et al. did not find an increased risk of VS in long-term mobile phone subscribers.[28] Further, although there is a preponderance of right-sided mobile phone users, VS is evenly distributed between left and right side.[7,28] Their findings were supported by the INTERPHONE study, a multicenter case-control study, and a population-based case-control study in Sweden.[29,30] Hence, it seems unlikely that use of mobile phone poses a major risk for developing VS. A similar conclusion has been made in relation to exposure to extremely low-frequency electromagnetic fields, a type of radiation omnipresent in modern society due to electric devices.[31] Studies on the association between exposure to noise and VS have also produced conflicting results, and many authors have raised the concerns regarding recall bias, selection bias and detection bias.[32-34] In a large case-control study that objectively estimated noise exposure, as opposed to self-reported noise exposure, no association to VS was found.[35] Other proposed risk factors include parous women, past diagnosis of epilepsy, history of hay fever and different occupational exposures, while tobacco smoking is associated with lower incidence of VS.[36-39] However, the biological effects of these factors remain unclear. Two studies have failed to demonstrate an association between viruses and VS.[40,41] However, they were both biased towards herpesviruses. In conclusion, there is no compelling evidence of an exogenous factor causing sVS today.

6.2 Genetics of neoplasms – cancer genetics

Neoplasm is a genetic disease, best understood as an evolutional process where cells acquire sequential aberrations in its DNA, conferring a selective growth advantage over other cells. A neoplasm might be benign or malignant, i.e., cancer, with local invasion and the ability to metastasize distinguishing the latter. In their seminal work, "Hallmarks of cancer", Hanahan and Weinberg defined the biological capabilities acquired by cells in order to become benign tumors and cancer.[42] They described 10 different tumorigenic capabilities which all lead to the same end goal, namely uncontrolled growth of cells, the defining feature of neoplasms (*Fig.3*). Early studies throughout the first half of the 20th century led to the notion that DNA aberrations were the substrate of neoplastic disease.[43] This was confirmed through pioneering work done in the 70s and 80s identifying the Philadelphia chromosome in chronic myelogenous leukemia, the transforming abilities of tumor DNA and point mutations in the oncogene *HRAS* causing urothelial cancer.[44-47] Ever since, our understanding of neoplastic disease as a genetic disease has evolved. All neoplasms are thought to share a similar pathogenesis in which a cell gains growth advantages over surrounding cells in a tissue through sequential acquisition of DNA aberrations leading to a clonal expansion of the transformed cell in a Darwinian manner.[48] The types of DNA aberrations acquired include single nucleotide variants (SNV), insertions and deletions of segments of DNA up to 1000 bp (indel), DNA rearrangements (e.g., translocations), copy number aberrations (CNA, i.e., loss or gain of DNA segments

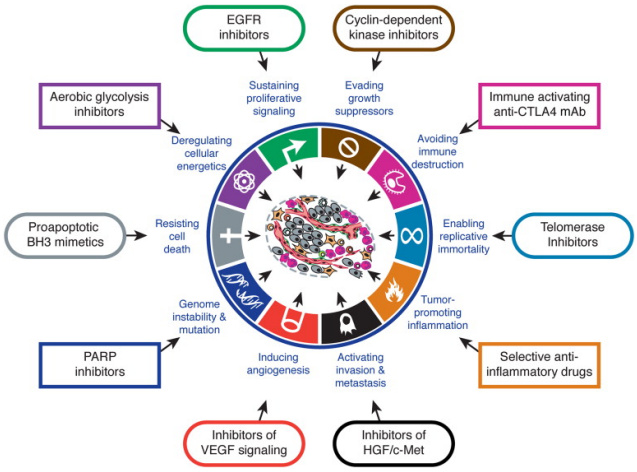

Figure 3. The hallmarks of cancer and possible therapeutics as depicted by Hanahan and Weinberg.[42]

larger than 1000 bp) and copy-number neutral regions of homozygosity (CNN-ROH, commonly referred to as loss of heterozygosity). To determine which DNA aberrations are causal in neoplasm is not trivial. This is in particular true in the genomic era, where new technologies, outlined below, generate vast amounts of data. Mutational processes operate during the lifetime of all cells, both due to endogenous and exogenous processes (e.g., carcinogens like tobacco smoke, UV radiation and tumor viruses). Distinguishing the numerous amounts of passenger mutations, i.e., mutations that does not give a selective growth advantage to the cell, from the driver mutations present in a neoplastic cell poses a major challenge.[49] Typically, driver mutations represent activating mutations in oncogenes (e.g., SNV, gene amplification and translocation) or inactivating mutations in tumor suppressor genes (e.g., SNV and deletion). The consequence of all driver mutations in neoplasm lies in giving the harboring cell a selective growth advantage.

6.2.1 Development of tools for genetic analyses

The rapid development of molecular technologies for analyzing nucleic acids evident today represents one of the major leaps in human history in the study of biology and disease. Since the identification of DNA as the substrate of inheritance, techniques have evolved from time-consuming procedures analyzing aneuploidy to analyzing the complete genome in single-nucleotide resolution in the manner of days.[50,51] Landmarks in this process include microscopy-based study of chromosomes (cytogenetics), color-labelling of DNA (molecular cytogenetics), microarray-based techniques, DNA sequencing, discovery of the DNA polymerase and the polymerase chain reaction (PCR) and the latest advent of next-generation sequencing (NGS). Sequencing, in this study, refers to determining the sequence of nucleotides in a DNA or RNA molecule. In 2001, the first complete human genome was published, paving the way for the genomics era.[52,53] In the following years, the availability of a reference genome, reduced cost of sequencing and increased availability of sequencing infrastructure rapidly increased the amounts of sequenced genomes. With the advent of next-generation DNA sequencing (NGS), number of sequenced cancer genomes exploded. This allowed for a comprehensive and unbiased view of the underlying genetics driving

cancers.[54] In the early 70's, Gilbert and Maxam spent two years sequencing 24 bp of DNA.[55] Today, 10^{12} bp of DNA can be sequenced in two days for a little over thousand dollars.[54] NGS was first used for whole-genome sequencing (WGS). However, as this was too expensive for most research groups at the time, methods for targeted sequencing utilizing NGS technology was developed, including the development of whole-exome sequencing (WES). WES involves a target enrichment (also called capture) step prior to sequencing where DNA probes, either printed on a microarray or in a solution, are used to extract DNA corresponding to all protein-coding regions of the genome (*Fig.4*). Most contemporary capture kits also include regulatory regions of the genome resulting in approximately 60 Mbp (6×10^7 bp), or 2% of the genome, being sequenced.

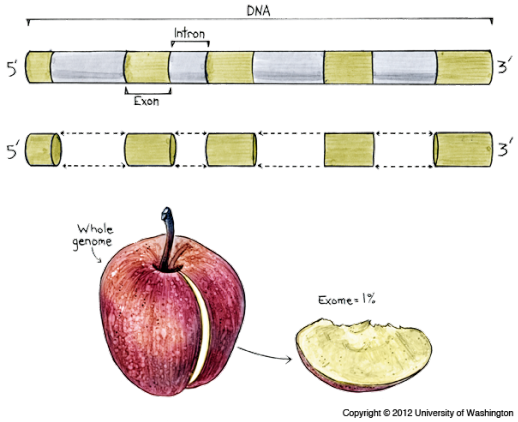

Figure 4. The exome is the DNA making up all the exons and comprises around 1% of the genome. Figure downloaded from https://mygene2.org/MyGene2/exomesequencingdetails

In the early days of DNA sequencing, analyzing the results involved large amounts of manual work, e.g., interpretation of radiographs from slab gels. However, with the enormous amount of data produced today, this is obviously unachievable. Hence, the development of genomic analysis techniques comes hand in hand with the development of bioinformatic tools. The output from a NGS sequencer is normally the nucleotide sequence of individual reads of a few hundred bp, along with quality parameter for each base and metadata about the sequence, namely the FASTQ format. A typical bioinformatic NGS workflow starts with aligning the individual reads stored in the FASTQ format to a reference genome using software like Burrows-Wheeler Aligner (BWA), resulting in a sequence alignment map (SAM) and its corresponding compressed binary (BAM).[56,57] In most cancer studies, one is interested in studying the

tumor-specific mutations. Hence, one BAM file is produced for both the tumor sample and a matched normal sample, typically from leukocyte DNA. The next step involves extracting the mutations present in tumor DNA, but not in normal DNA, in a process named variant calling, resulting in a variant call format file. Several quality check procedures are included in all steps, and automated workflows, incorporating all steps from raw sequence reads to called variants, have been developed.[58] The biggest challenge in utilizing NGS data lies in identifying the driver mutations among the numerous passenger mutations.[49] Different strategies exist for this purpose including: 1) frequency-based, i.e., identifying recurrent mutations or recurrently mutated genes in a cohort; 2) prediction of functional impact of the mutation, e.g., mutation in an evolutionary conserved region and missense mutations resulting in amino acids with marked different physicochemical properties; 3) identifying signaling pathways with more mutated genes than expected.[59-62] The resulting candidate variants are then typically visualized and quality checked manually through the use of software like Integrative Genomics Viewer (*Fig.5*).[63] Bioinformatic methods can only go as far as suggesting candidate cancer driver genes, but has proved valuable in prioritizing variants for functional assays. Perturbing genes in functional assays, like cell culture or animal models, and observe phenotypic changes leading to a selective growth advantage, remains the gold standard for identifying cancer drivers. Although this has traditionally been labor-intensive work, recent advances have paved the way for high-throughput functional assays.[64] However, as more and more cancer drivers are characterized and made available in public

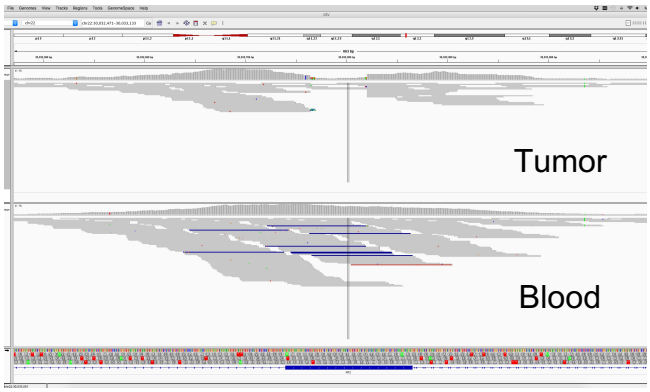

Figure 5. DNA sequences (reads) from WES in tumor and matched blood aligned against an exon of NF2 (blue bar at the bottom), visualized in Integrative Genomics Viewer.[62] Here we see a lack of reads in tumor aligning against this exon, indicating an exonic deletion in NF2.

databases, like COSMIC, one can circumvent the need for functional assaying in every cancer genetics study.[65]

6.2.2 Clinical utility of cancer genetics

A major promise of cancer genetics is finding druggable driver mutations in neoplastic disease. An early example of this was the finding of the Philadelphia chromosome (a chromosomal translocation resulting in a *BCR-ABL* fusion producing a constitutively active tyrosine kinase) in nearly all patients with chronic myeloid leukemia, the subsequent development of a specific inhibitor (Imatinib) of the gene product resulting in a dramatic drop in mortality.[66] Other notable examples include targeting *BRAF* in melanoma, *PARP1* in breast cancer, *EGFR* in lung cancer and *ALK* in lung cancer.[67-70] Efforts have been made to make all data on druggable driver mutations available for clinicians, in part realizing the potential of personalized cancer treatment.[71]

Cancer genetics has also supplemented and in some cases transformed the diagnostic procedure and prognostics of some common types of cancers. Most notably in the area of gliomas, where mutational status of *IDH1/2*, chromosome 1p/19q and *TP53* predict prognosis better than standard histology-based diagnosis.[72] Other areas where cancer genetics have benefited the care for cancer patients include detection of residual disease and cancer prevention.[73,74]

In the post-genomic era, large international consortiums and projects have harnessed the opportunities inherent in NGS to characterize numerous types of cancers.[75,76] In addition to vastly increasing our understanding of the biology of neoplastic disease, this has accelerated the pace of the discovery of druggable targets.[77] Initial studies demonstrated the feasibility of WES in characterizing all SNV and indels in the coding region of a tumor genome (the exome), whereas WGS could analyze the full spectrum of mutations.[76,78-80] The NGS era has culminated in the impressive work from ICGC/TCGA Pan-Cancer analysis of whole genomes consortium, presenting an integrative analysis of 2658 cancer genomes with matched normal, and making all data available to the scientific community.[81] However, personalized cancer treatment has yet to profit most cancer patients. Translation of the vast amount of data on cancer

genomes to everyday clinical practice, is mainly hampered by our limited understanding of the function of the genetic alterations discovered.[77] Hence, there is a call for international collaborations to perform high-throughput functional assays, with the aim to perturb all known cancer mutations in all types of cancers in different stages, termed the "Cancer Dependency Map", with the opportunity to fully realize the potential of personalized genomics-guided cancer treatment.[82]

6.2.3 Genetics of vestibular schwannoma

As outlined above, all neoplasms are caused by heritable alterations, genetic or epigenetic, providing a selective growth advantage to the harboring cell. Heritable, in this setting, refers to alterations passed on through mitosis in somatic cells. This is also true for VS. Neurofibromatosis type 2 (NF2) is an autosomal dominant multiple neoplasia syndrome characterized by bilateral VS.[83] Early genetic linkage studies found the gene responsible for NF2 to be located on chromosome 22q12.[84] Subsequent studies identified the novel tumor suppressor gene *NF2* (neurofibromin 2), encoding the merlin protein, and also linked this gene to sVS.[85,86] Since its initial identification, several studies have characterized the spectrum of *NF2* mutations in sVS.[87-91] Further, immunological studies on the merlin protein found downregulation and absence of the protein in most or all tumors, even those lacking biallelic inactivation of *NF2*.[89,92,93] Other possible mechanisms for disrupting merlin function, besides intragenic mutations, include CpG methylation in the promoter region of *NF2*, post-translational regulation by regulatory RNAs (e.g., microRNA) and non-coding variants.[94-96] Merlin acts as a tumor suppressor at the cell membrane by mediating contact-dependent inhibition of proliferation and in the nucleus by binding a E3 ubiquitin ligase.[97,98] Although *NF2* is a well-established tumorigenic factor in VS, some lines of evidence suggest that other factors also contribute: 1) the type of *NF2* mutation does not seem to correlate with tumor behavior; 2) some tumors lack *NF2* mutation; 3) a single driver mutation is rare in neoplasm.[81,99-103] Hence, there has been put some effort into elucidating other genetic determinants of VS tumorigenesis and clinical behavior.

The development of microarray technology has enabled the characterization of the VS transcriptome, i.e., all the transcribed genes in a biopsy. This has elucidated a

number of deregulated genes and signaling pathways in VS compared to normal nerve tissue, including *CAV1*, *PTEN*, *MET* pathway and *ERK* pathway.[100,104,105] One notable finding was that the *PI3K/AKT/mTOR* pathway was upregulated in VS and that pharmacological inhibition suppressed tumorigenesis in cell culture.[106] Microarray technology and comparative genomic hybridization have been used to probe the VS genome for CNA and CNN-ROH. The only consistent finding across these is loss of chromosome 22q, although other candidate regions have been reported by the different groups.[107-109]

With the emergence of NGS technology, some groups have harnessed the technology to assess the genetics of VS. In the first NGS study on VS, in 2016, Agnihotri et al. used WES on 26 schwannomas (13 VS and 13 spinal schwannomas) to find a mean of 17 somatic exonic mutations.[110] They confirmed *NF2* mutation as the main driver event, but also found novel recurrent mutated genes, including *ARID1A*, *ARID1B* and *DDR1*. Interestingly, they used RNAseq (whole-transcriptome sequencing) and RT-PCR to identify a novel fusion gene, *SH3PXD2A-HTRA1*, in 12/125 (10%) of the samples. The product of this fusion demonstrated tumorigenic properties, which were reversed in vitro upon pharmacological inhibition. In 2018, Carlson et al. used a combination of WES, RNAseq and WGS to, for the first time, find biallelic inactivation of *NF2* in all samples analyzed (23 sVS).[111] The number of somatic mutations ranged from 26-72, but no details on the non-NF2 mutations were provided. The study cohort was biased towards clinically aggressive tumors (i.e., fast growth, large tumors, cystic tumors and recurrent tumors) to correlate genetic findings with clinical behavior. Indeed, they found that large CNAs other than chromosome 22 loss, correlated with a more aggressive phenotype. A similar conclusion was reached in a WGS study on a cohort of 10 familial VS.[112] Aaron et al. used a combination of WES and RNAseq to profile 12 VS, including 8 cystic tumors and 2 previously irradiated tumors, to find a similar number of somatic mutations as previous NGS studies.[113] However, one previously irradiated tumor harbored 184 mutations. No new recurrent gene was found, but they postulated that genome-wide CNA patterns correlated with cystic tumors and previous radiation-therapy. Further, Evans et al. demonstrated, in their study on de novo NF2 cases, the utility of NGS in detecting

variants with low frequency.[114] In conclusion, except from disruption of *NF2*, no other consistent genetic finding has been found in VS. However, it seems likely that other genes contribute to a subset of VS.

6.3 Gamma knife radiosurgery

Gamma knife radiosurgery (GKRS) (Elekta AB, Stockholm, Sweden) is a type of SRS used for the treatment of intracranial lesions, including VS (*Fig.6*). Ionizing radiation (IR) in the form of gamma rays from 192 radioactive Cobalt-60 sources, evenly distributed in a treatment helmet, converge to produce high radiation dose in a focal point, whereas the deposited radiation in the surrounding healthy tissue remains low. Standard treatment protocols for VS typically delivers 12-13 Gy to the margins of the tumor. GKRS has become an important treatment alternative for small- to medium-sized VS with high rates of tumor control and low rates of complications.[20]

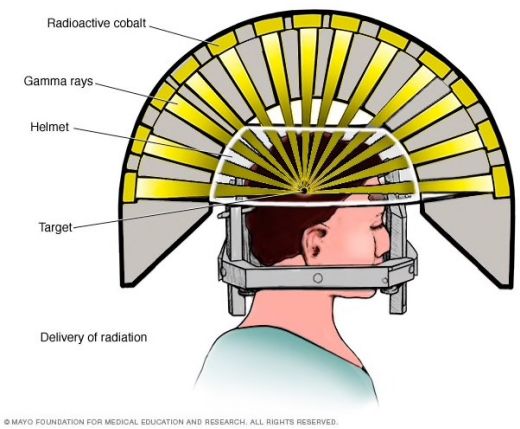

Figure 6. Gamma knife radiosurgery. Figure downloaded from https://www.mayoclinic.org,

6.3.1 Radiation-induced neoplasia

Radiation-induced neoplasia (RIN) is a well-established phenomenon, and evidence for it has accumulated from both epidemiological, experimental and genomics studies.[115] Already in 1948, in its seminal paper on radiation-induced sarcoma, Cahan et al. outlined the evidence suggesting RIN, now known as the Cahan's criteria: 1) the tumor must arise within a previously irradiated tissue; 2) histological and radiographic examination must confirm a different diagnosis at recurrence; 3) a latency period of > 5 years from radiation therapy to RIN; and 4) the patient must not have a tumor

predisposing syndrome.[116] The linear no-threshold model is used to describe the linear dose-response relationship between IR and cancer risk.[117,118] Although disputed, the consequence of this model is that there is no safe limit for exposure to IR, and that even small amounts increase the risk accordingly. Early evidence demonstrated the role of large amounts of radiation, experienced by e.g., atomic bomb survivors, in causing cancer. However, recent studies indicate the role of medical radiation, in the form of radiotherapy and diagnostic radiography, in causing cancer.[119]

Amid increasing use of GKRS in the treatment of VS, several groups have presented cases of malignant transformation and second malignancies following radiation, hence questioning its safety.[120-126] The risk of this is increased in patients with tumor prone syndromes, like NF2.[127] However, epidemiological studies suggest that the risk of GKRS-induced malignant transformation and second malignancies remains low in patients with sVS.[128-130] A recent retrospective multicenter cohort study, including 4905 patients treated with stereotactic radiosurgery for intracranial lesions with a median follow-up of 8.1 years, concluded that the incidence of secondary malignancies and malignant transformation in GKRS treated patients was similar to the general population.[131] Similarly, in an analysis of 9460 patients treated for sVS, there was no difference in the incidence of a second intracranial neoplasm between the GKRS- and microsurgery-treated patients, further weakening the role of GKRS in inducing secondary neoplasm.[132]

6.3.2 Ionizing radiation and its effects on DNA

Neoplasia is a disease of the genome, and hence, the carcinogenic potential of IR lies in its interaction with DNA. Energy is deposited as the radiation traverse human tissue and might damage the DNA directly through single strand or double strand breakage, or indirectly through producing reactive oxygen species.[133] The result depends on, among other factors, the radiation dosage and the affected cell's DNA repair mechanisms.[134-136] Any type of mutation might be induced.[137] However, several studies have identified different mutational signatures associated with IR. In a WGS study on 12 radiation-associated malignancies (sarcomas and breast cancer) and 319 radiation-naïve malignancies, a signature based on an excess of small deletions and

balanced inversion was found to be associated with IR.[138] These findings were to some extent confirmed in WGS data from a large number of organoids from different tissues, both human and mice, experimentally radiated with gamma-ray or x-ray, as well as 22 human RIN.[134] In addition to indels and structural variants, complex genomic rearrangements, e.g., chromothripsis, were found in 30% of irradiated cells but in none of the radiation-naïve cells. It was estimated that 16 mutations were induced for every 1 Gy of IR. Interestingly, the lesions were distributed evenly throughout the genome and did not differ with replication timing, sequence context or chromatin structure, consistent with the random nature of IR in damaging DNA.

6.3.3 Radiosensitivity

A small proportion of sVS, around 5 %, are radioresistant, i.e., they do not respond to GKRS treatment.[20] Already in 1981, Anniko et al. exposed VS tissue in vitro to gamma irradiation to find that, in some specimens, viable cells existed even after doses up to 150 Gy.[139] They further speculated that the variable radiosensitivity relied upon different repair capacity and that cells in interphase, i.e., not replicating, were radioresistant to a higher degree. Lee et al. found that 20/26 radiation-naïve VS and 0/4 irradiated VS demonstrated LOH on chromosome 22q.[140] However, none of the irradiated VS expressed merlin, leading them to postulate that alternative mechanisms for *NF2* inactivation correlated with radioresistance. Hansen et al. demonstrated that 10 Gy of radiation induces DNA double-stranded breaks in cultured VS cells and that the proportion of apoptotic cells following radiation is correlated with proliferation rate.[141] Hence, they proposed that radioresistance in VS likely is due to low proliferative capacity rather than increased resistance to DNA damage. However, a recent study found that cMET signaling was upregulated in radioresistant schwannoma and that inhibition of cMET increased radiosensitivity by enhancing DNA damage.[142] Upregulated PD-L1 protein, a known negative regulator of the immune-mediated anti-tumor response, was shown to correlate with radioresistant VS, lending support to a role of immune evasion in GKRS failure.[143] However, it is unclear whether upregulated PD-L1 is a consequence of IR or that it is the cause of radioresistance. It has been hypothesized that upregulated VEGF (vascular endothelial

growth factor, a stimulator of angiogenesis) rescues tumor associated endothelial cells in response to IR, leading to radioresistance in some VS.[144] In agreement with this, inhibition of VEGF potentiates the effect of IR on mouse schwannoma.[145] Interestingly, anti-VEGF therapy normalized tumor vasculature and increased perfusion, thereby sensitizing mouse schwannoma cells to IR by increasing tissue oxygenation. IR has been shown to induce oxidative stress in VS that recur despite being treated with both surgery and IR.[146] Some evidence suggests that persistent c-Jun N-terminal kinase activity is responsible for scavenging reactive oxygen species following IR and that inhibition increases radiosensitivity in VS.[147] The molecular mechanism underlying radiosensitivity in other neoplastic disease has been elucidated in some cases and often relates to altered DNA repair mechanisms.[148] Skinner et al. demonstrated that gain of function mutations in *TP53*, a tumor suppressor involved in DNA repair, correlated strongly with radioresistance in head and neck squamous cell carcinoma.[149] Another study found that loss of function mutations in *ATM*, also involved in DNA repair, associated with exceptional responders to radiotherapy.[150] Although changes in the transcriptome of irradiated VS have been identified, no genetic biomarker of radiosensitivity has been identified.[151]

6.4 Malignant transformation of VS

Malignant peripheral nerve sheath tumor of the vestibulocochlear nerve (VN-MPNST) is exceedingly rare and carries a poor prognosis.[152] Approximately half arise de novo while the rest are thought to occur as a result of malignant transformation from VS.[153] A history of radiation exposure and neurofibromatosis type 1 or 2 are known risk factors. A review by Seferis et al. in 2014 identified 29 cases of VN-MPNST after radiation therapy and 30 cases of malignant transformation in the absence of radiation, but few of the cases had histologic evidence of transformation.[125] Carlson et al. recently, in a review of radiation-naïve VN-MPNST, estimated that one VN-MPNST occur for every 1041 VS.[154] The most recent review found 71 cases of malignant transformation of VS in the literature.[152]

6.4.1 Genetics of MPNST

To the best of our knowledge, no studies exist assessing the genetics of a VS undergoing malignant transformation. However, this process has been elucidated in some cases of malignant transformation of a neurofibroma in the setting of neurofibromatosis type 1, where homozygous loss of the tumor suppressor *CDKN2A* has been suggested as an initiating event.[155,156] Only two cases of VN-MPNST have been genetically characterized, using array comparative genomic hybridization to identify a large number of CNAs.[157] This is in line with genetic studies on extracranial MPNST and other soft tissue sarcomas, which are dominated by extensive CNAs and relatively low burden of small mutations.[158,159] Driver mutations identified in extracranial MPNST include amplifications of *IGF1R* and *EGFR*, deletion of *CDKN2A* and small mutations in *NF1* and *TP53*.[160,161] Recent studies, utilizing NGS technology, have identified recurrent mutations in members of the Polycomb repressive complex 2.[162-164] However, except from single gene analysis in case reports and the aforementioned study analyzing for CNA, assessment of the VN-MPNST genome at single nucleotide resolution has not been undertaken.[121]

7. Aims of present study

7.1.1 Overall aim

The aim of this thesis was to characterize the genome of sporadic vestibular schwannoma and malignant peripheral nerve sheath tumor of the vestibulocochlear nerve with a special emphasis on tumors exposed to ionizing radiation.

7.1.2 Study I

To identify tumor specific mutations in the coding region of the sVS genome, i.e., the exome, and to compare the mutational spectrum in tumors treated with GKRS to radiation-naïve tumors.

7.1.3 Study II

To identify viral nucleic acids in sVS biopsies.

7.1.4 Study III

To identify genome-wide tumor specific copy number aberrations in sVS, compare the genome of tumors treated with GKRS to radiation-naïve tumors and infer intratumoral heterogeneity.

7.1.5 Study IV

To identify tumor specific mutations associated with malignant transformation of sVS in the absence of ionizing radiation, present comprehensive genomic analyses of three cases of the extremely rare cancer VN-MPNST and compare irradiated to radiation-naïve VN-MPNST.

8. Methods

8.1 Patients and tissue sampling procedures

Tumor specimen and matched blood sample from patients undergoing VS surgery has been collected prospectively since 2001 at the Department of neurosurgery, Haukeland University Hospital, as part of the Bergen neurosurgical tissue bank. In 2012, Haukeland University Hospital was designated the National treatment center for vestibular schwannoma, and since, all patients in Norway with diagnosed VS are referred to this tertiary care center. Tissue is harvested from the subcapsular part of the tumor through retrosigmoid craniotomy. From 2001 – 2010, Dexamethasone 4 mg x4 was given orally one day prior to surgery. However, this routine was abandoned, and Dexamethasone is now only given if increased posterior fossa pressure is anticipated. All procedures are carried out by one of three consultant neurosurgeons specialized in VS surgery. The specimens are either snap frozen in liquid nitrogen at the surgical theatre or stored at -80 degrees before transfer to a designated liquid nitrogen tank at the Center for medical genetics and molecular medicine, Haukeland University Hospital. Clinical data are recorded prospectively, and routine histopathological examination are performed at the Department of pathology by neuropathologists in all cases. In study 3, volumetric tumor measurements were performed on BrainLab Elements if preoperative MRI scans were available (Version 2.4.0, BrainLab AG, Munich, Germany). In studies 1, 2 and 4, maximal extrameatal tumor diameter was used to represent tumor size. In studies 1 and 3, the study participants were biased towards patients failing GKRS treatment to include all tumors exposed to ionizing radiation. GKRS is performed according to a standardized protocol in a single session with a marginal dose of 12 Gy. In study 2, tibial nerves served as normal controls. In study 4, three patients with VN-MPNST were included. Two patients were primarily treated with GKRS at our institution for a presumed VS, progressed after 9 and 12 years and were operated for VN-MPNST. In addition, as part of a cooperation, tissue from a patient operated for VS and, subsequently VN-MPNST at Department of Neurosurgery at Rigshospitalet, Copenhagen, Denmark, was included. This patient was first operated

for a VS and progressed to VN-MPNST in the absence of radiation as previously described.[165] Written informed consent was obtained from all patients prior to surgery, and the study was approved by the Regional Ethical committee for medical research in Western Norway (2013/374).

8.2 DNA analyses

8.2.1 DNA extraction

DNA was used in all studies. First, tumor tissue was disrupted using the TissueLyser (Qiagen) and treated with protease. Next, DNA was extracted using the QIAamp DNA Mini Kit (Qiagen). DNA from blood was extracted using QIAsymphony (Qiagen). The DNA quality and quantity were evaluated with 1% SeaKem gel electrophoresis and NanoDrop spectroscopy (Thermo Fisher Scientific), respectively.

8.2.2 Whole-exome sequencing

WES was used to analyze tumor and matched blood DNA in 46 and 3 patients in studies 1 and 4, respectively. The sequencing was done either as a custom service at HudsonAlpha Institute for Biotechnology or at our institution. Capture kit was NimbleGen SeqCap EZ Exome Library v3.0. Paired-end sequencing (2 x 100 bp) was executed on Illumina HiSeq to an approximately 85 x coverage. The resulting reads were aligned to the hg19/GRCh37 reference genome using Burrows-Wheeler transform.[56] Postprocessing of the alignments were done using GATK v3.2 and SNVs and indels were called using a combination of GATK haplotype caller and MuTect.[58,166] ANNOVAR was used to annotate the variants.[61] To ensure a high-confidence list of variants, a number of filtering steps were done, and the variants were manually checked in Integrative Genomics Viewer (IGV).[63] For details on the filtering process, see the respective papers. In study 4, MuSiCa was used to infer the contribution of the COSMIC mutational signatures in all WES analyzed tumors, including 47 VS and 3 VN-MPNST.[167] In study 3, WES data available from 46 VS in study 1 were analyzed

in Sequenza to estimate aberrant cell fraction and allele-specific copy number profiles.[168]

8.2.3 Whole-genome sequencing

In study 2, we subjected tumor DNA from two patients to WGS to detect any viral DNA present. Paired-end (2 x 125 bp) sequencing was done on Illumina HiSeq to approximately 30 x coverage. The resulting reads were aligned to hg19/GRCh37 reference genome using Bowtie 2.[169] The unaligned reads were then aligned to all known viral sequences downloaded from National Center for Biotechnology Information in August 2016. All reads aligned to a viral genome were counted and manually reviewed in IGV.

8.2.4 Whole-genome DNA microarray

CytoScan HD microarray (Affymetrix, UK), a whole-genome DNA microarray able to detect chromosomal aberrations > 25-50 kbp, was used in study 3 and 4 to analyze tumors in 27 and 3 patients, respectively. Tumor DNA labelled with fluorescent dye is hybridized to a microarray consisting of 2.7 million probes, including 700'000 polymorphic probes and 2 million nonpolymorphic probes. For study 4, matched normal DNA was included as controls. After hybridization, the microarrays are scanned to produce fluorescence intensity files (CEL files) that indicate the amount of DNA hybridized to each probe. The intensity files are normalized and processed through three different variant calling software: 1) ChAS v3.2 (Affymetrix, UK), 2) Rawcopy and 3) Nexus Copy Number (BioDiscovery, USA).[170] A per-sample union variant call list is produced listing CNA and CNN-ROH present in each sample. These variants are filtered through several steps to produce candidate variants as detailed in the respective papers. Raw probe level data from candidate CNAs and CNN-ROHs, i.e., b-allele frequency (from polymorphic probes) and log ratios (amounts of DNA at each probe in tumor relative to normal controls), are imported and manually reviewed in IGV. GISTIC was used to identify significantly aberrated regions throughout the cohort and ASCAT was used to infer aberrant cell fraction and absolute allele-specific copy number of each sample.[171,172] In study 4, called variant segments were imported

to CTLPscanner and analyzed to identify regions of chromothripsis-like patterns (CTLP) in the tumors.[173]

8.2.5 MLPA

In study 1, we used multiplex ligation-dependent probe amplification (MLPA) of the complete *NF2* gene in 46 sVS.[174] This method complements WES by reliably detecting intragenic and whole-gene deletions or duplications. The SALSA MLPA probe mix P044-B2 NF2 was used, and data was analyzed in Coffalyzer (MRC-Holland). Normal controls included chorionic villi DNA and leukocyte DNA from blood donors, according to standard procedure at our institution.

8.2.6 Microsatellite instability PCR

In study 1, one patient demonstrated a large number of tumor specific mutations. Hence, we decided to investigate for the presence of microsatellite instability, a marker for defective DNA mismatch repair system found in other neoplastic disease.[175] For this purpose, standard procedures with PCR amplification and capillary electrophoresis of the mononucleotide markers NR21, NR24, NR27, BAT25 and BAT26 was done as previously described.[176]

8.3 RNA analyses

8.3.1 RNA extraction

For study 2, we used the Qiagen RNeasy minikit (Qiagen, Hilden, Germany) to extract total RNA. The RNA quality and quantity were evaluated on the BioRad Experion (BioRad, Hercules, California) and NanoDrop (Thermo Fisher Scientific, New York, New York), respectively.

8.3.2 ViroChip

ViroChip is an RNA microarray with 60’000 probes designed to hybridize to all known viruses.[177] This allows for a complete unbiased characterization of all viral transcripts present in a tissue. In study 2, RNA from 15 sVS and one normal nerve

control was hybridized to ViroChip as a custom service at the UCSF Viral Diagnostics and Discovery Center, San Francisco, CA, USA. Signal intensity files were normalized and analyzed using two different algorithms, the Z-score and the E-predict.[178,179] See paper 2 for details. This results in a list of candidate viruses present in the tumors.

8.3.3 Whole-transcriptome sequencing

To complement and verify the results from ViroChip, we subjected RNA from seven sVS and one normal nerve to RNAseq in study 2. A combination of poly-A enrichment and total RNA enrichment was used to capture viral transcripts lacking polyA-tail on mRNA. Paired-end sequencing (2 x 75 bp) was done on Illumina HiSeq by HudsonAlpha or Nextseq 500 at our institution to a total of approximately 100M reads. The resulting reads were processed as described in Whole-genome sequencing.

8.3.4 RT-PCR and qRT-PCR

An extended set of 46 sVS and four normal nerves were analyzed with PCR-based methods to verify results from ViroChip in study 2. The strongest hits from ViroChip were selected and included Human Herpesvirus 1 and 2 and Human Endogenous Retrovirus K. Details on primer sequences, probes, controls and reaction conditions are described in paper 2. Results from RT-PCR were analyzed using gel electrophoresis. qRT-PCR was performed on the ABI 7900 instrument (Applied biosystems), β-actin was used to normalize the data and the relative expression in tumor compared to normal nerve was quantified using the $2^{-\Delta\Delta Ct}$ method.[180]

8.4 Statistical analyses

Statistical analyses in all studies, including descriptive statistics, contingency table statistics, Mann-Whitney U test and linear correlation, were done using R, Microsoft Excel and/or Nexus Copy Number.[181] Pathway analysis and gene ontology annotations were done with Ingenuity Pathway Analysis (Qiagen Inc.) and The Reactome Knowledgebase in study 1 and 4, respectively.[182] In Ingenuity Pathway Analysis, P-values were calculated using the Fisher's exact test and p-values < 0.05

were considered significant. This is a measure of the probability that a set of genes are associated with a specific pathway by chance alone. In the Reactome Knowledgebase, p-values are calculated through the binomial test, and together with the False Discovery Rate calculated using the Benjamini-Hochberg approach, helps determining whether more genes are mutated in a pathway then expected by chance. In studies 3 and 4, hierarchical clustering based on copy number profiles were done using the hclust function in Rawcopy and the built-in complete linkage hierarchical clustering algorithm in Nexus Copy Number. In study 4, unsupervised hierarchical clustering and principal components analysis of the mutational signatures were done in MuSiCa.

9. Summary of results

9.1 Study 1: Genetic landscape of sporadic vestibular schwannoma.

To characterize the genetic landscape of sVS, we performed WES on 46 tumors and matched blood samples, including 8 previously irradiated tumors. This provided an unbiased view of the full exonic tumor-specific mutational spectrum. Mean age across the cohort was 51.3 years, 52% were female and mean tumor size was 31 mm. Excluding one outlier, a total of 716 mutations were found, including 676 SNVs and 40 indels. A median of 14 (4 - 57) genes were mutated. The outlier harbored 231 mutations, including in the DNA repair gene *RAD54L*, and was also an outlier clinically with high age, treatment resistance and rapid growth. Notably, the irradiated tumors were not hypermutated (*Fig.7*). The most significantly mutated gene across the cohort was *NF2*. By combining the results from WES and MLPA, 35 tumors (76%) had mutated *NF2*, including 16 tumors (35%) with both alleles mutated. In total, we identified 30 SNVs, 15 indels and 11 larger deletions, with a preponderance of loss-of-function mutations in *NF2*. Variant allele frequency varied from 8% to 69%, indicating the coexistence of different tumor clones or infiltrating normal cells. The tumors with wild-type *NF2* alleles harbored mutations in genes that could be linked to merlin function. Two other novel genes were identified in a subset of sVS, *CDC27* in five patients (11%) and *USP8* in

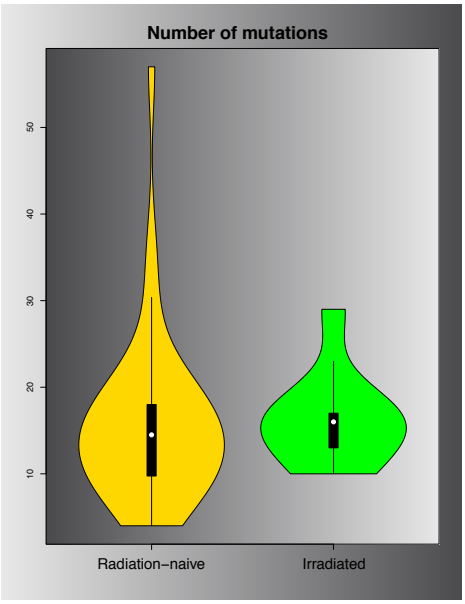

Figure 7. Violin plot demonstrating that the number of tumor specific mutations (y-axis) is similar in irradiated and radiation-naïve sVS.

three patients (7%). We found another 16 genes mutated in two tumors: *CHD4, CTAGE6, CTNNA2, EIF5B, HS6ST1, KALRN, LGR5, LGSN, NAV3, OR2T3, PKD1, PLEC, POTEJ, RAD54B, TENM2* and *TTN*. 12 patients had mutations in genes coding for proteins with known pharmacological inhibitors. Through pathway analysis we identified the axonal guidance pathway to be significantly mutated (p-value 5.36 x 10^{-5}), with 25 tumors (54%) harboring a mutated gene in this pathway. We found no genes or mutated pathways that could be associated with previous radiation exposure. No tumor demonstrated microsatellite instability, indicating normal functioning mismatch DNA repair.

9.2 Study 2: Screening for viral nucleic acids in vestibular schwannoma.

To screen for viral nucleic acids in sVS, we used complimentary unbiased transcriptomic and genomic approaches as well as confirmatory PCR-based methods on RNA and DNA from tissue biopsies. The 15 patients included in the ViroChip analysis had a mean age of 49 years, 47% were female and mean tumor diameter was 31 mm. The first screening utilizing ViroChip indicated that several human and non-human viral transcripts were present. However, most could not be verified in follow-up analyses. One virus, Human Endogenous Retrovirus K (HERVK), was identified in all samples both at DNA and RNA level. WGS data indicated that several copies of the HERVK genome was present in the sVS genome, with an approximate 300 x coverage of the viral genome as compared to the average coverage of 30 x across the sVS genome (*Fig.8*). However, HERVK transcripts

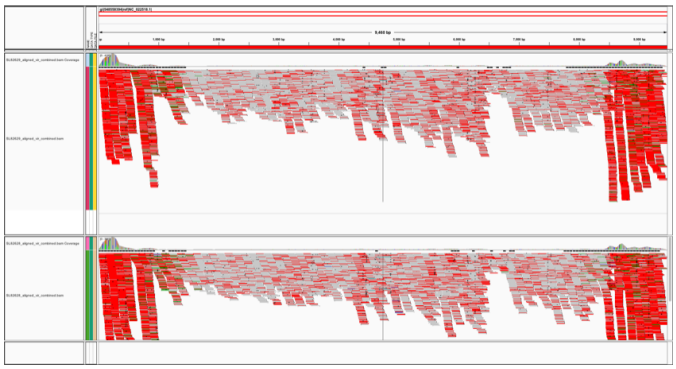

Figure 8. Reads from WGS aligned to the genome of HERVK as visualized in Integrative Genomics Viewer.

were also expressed in the normal nerves and expression level did not differ between tumor and normal (*Fig.9*). Sequencing of the whole transcriptome (RNAseq) did not reveal any other viruses than HERVK. Through WGS, we identified small amounts of sequences mapped uniquely to the Epstein-Barr virus, indicating small amounts of this virus present in both samples analyzed.

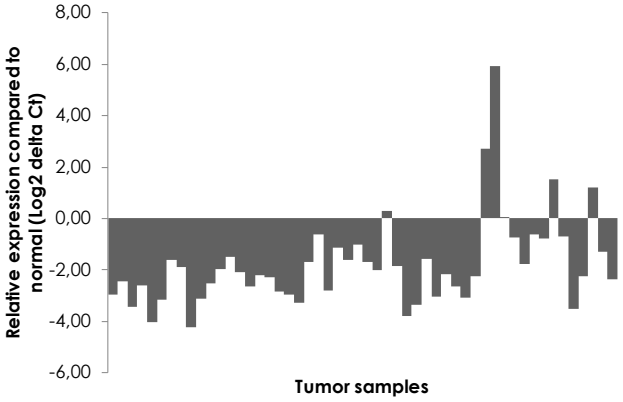

Figure 9. Expression of HERVK transcripts in sVS relative to expression in normal nerves as measured by RT-PCR. Most sVS express lower levels of HERVK RNA with a few outliers with higher expression.

9.3 Study 3: Gamma Knife Radiosurgery does not alter the copy number aberration profile in sporadic vestibular schwannoma.

To complement the genetic characterization of sVS and irradiated VS from study 1, we analyzed a total of 55 sVS, including 18 irradiated, for CNA and CNN-ROH. A combination of whole-genome DNA microarray and WES was used for this purpose. Mean age at the time of surgery was 53.3 years and mean preoperative volume was 8.4 cm^3. The genome of sVS was fairly diploid, with a median of 7 (0-58) CNAs identified covering a median of 0.17% of the sVS autosome (chromosomes 1-22) (*Fig.10*). Gains were more common than losses, with a median ratio of 1.25 (0.25 – 6.50). A total of 38 genomic loci was identified as significantly mutated. However, except from chromosome 22q aberrations, all were normal variants or non-coding variants. Loss or CNN-ROH of chromosome 22q, where *NF2* resides, were identified

in 25/55 (45%) patients and was highly significant by GISTIC analysis (Q-bound = 1.36 x 10^{-9}, G-score = 15.72). Among the tumors profiled with both microarray and WES, we found that 89% of sVS harbored at least one mutated *NF2* allele and 44% harbored two mutated alleles. We estimated aberrant cell fraction to vary from 25% - 94%, indicating the presence of several distinctive clones. However, we did not find two or more aberrations with differing variant allelic fraction within the same tumor, indicating that the coinciding clone was not aberrant. Aberrant cell fraction did not correlate with tumor growth. By hierarchical clustering, copy number profile was not associated with previous radiation exposure. No aberrated loci was found to correlate with previous radiation exposure.

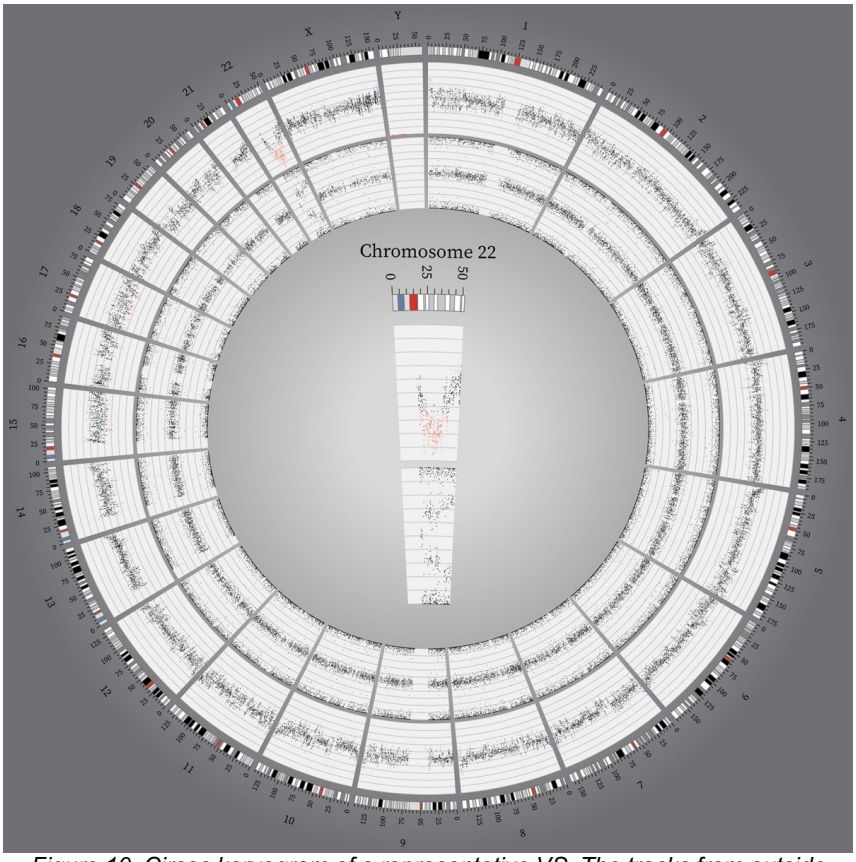

Figure 10. Circos karyogram of a representative VS. The tracks from outside inwards: chromosome numbers, chromosomal position in Mbp, copy number and allele patterns. Most chromosomes show a normal diploid pattern. Highlighted in the middle is chromosome 22 where NF2 resides. It is affected by a mosaic loss followed by a CNN-ROH.

9.4 Study 4: Genetic alterations associated with malignant transformation of a sporadic vestibular schwannoma.

In this study we were able, for the first time, to assess the genomic changes associated with malignant transformation of a sVS. Matched sVS, VN-MPNST and blood sample from one patient were analyzed with a combination of whole-genome DNA microarray and WES. The patient, a then 47-year-old female, presented in 2010 with unilateral hearing loss, tinnitus, balance disturbance and unilateral facial numbness and was operated with near-total resection of a VS as previously described.[165] In 2014 she presented again with facial paralysis and growth of the tumor remnant and underwent a second surgery. Histopathological examination and immunohistochemistry demonstrated that the tumor had transformed to VN-MPNST in the absence of radiation exposure. Notably, the sVS demonstrated a grossly aberrated genome with CNAs affecting a total of 45% of the genome, in stark contrast with the results in study 3. A homozygous loss of the tumor suppressor gene *CDKN2A* and CTLP of chromosome 7 were present already in the sVS, and also retained after transformation. Average ploidy was 1.72 in sVS and 3.96 in VN-MPNST, indicating a whole-genome doubling occurring through the malignant transformation. 22 and 47 small mutations (SNVs and indels) were identified in the sVS and VN-MPNST, respectively. Notably, the sVS harbored chromosome 22 loss and a nonsense SNV (p.Q79X) in the remaining *NF2* allele that was not found in the VN-MPNST. Other small mutations found in VS were retained in VN-MPNST. Mutations in three COSMIC cancer census genes were acquired in the VN-MPNST: *GNAQ* (p.T96S), *FOXO4* (p.S71C) and *PDGFRB* (p.V568E).

To characterize the genome of VN-MPNST in a larger cohort, further two patients with VN-MPNST were included. Both had been primarily treated with GKRS, and hence lacked tissue from premalignant state. This enabled us to compare the irradiated to the radiation-naïve VN-MPNST. All tumors were characterized by grossly aberrated karyotype and a relatively modest number of small mutations, with 36-81% of the genome affected by CNA and 41-49 small mutations identified (*Fig.11*). Homozygous deletion of *CDKN2A* was present in all tumors. CTLP of chromosome 7

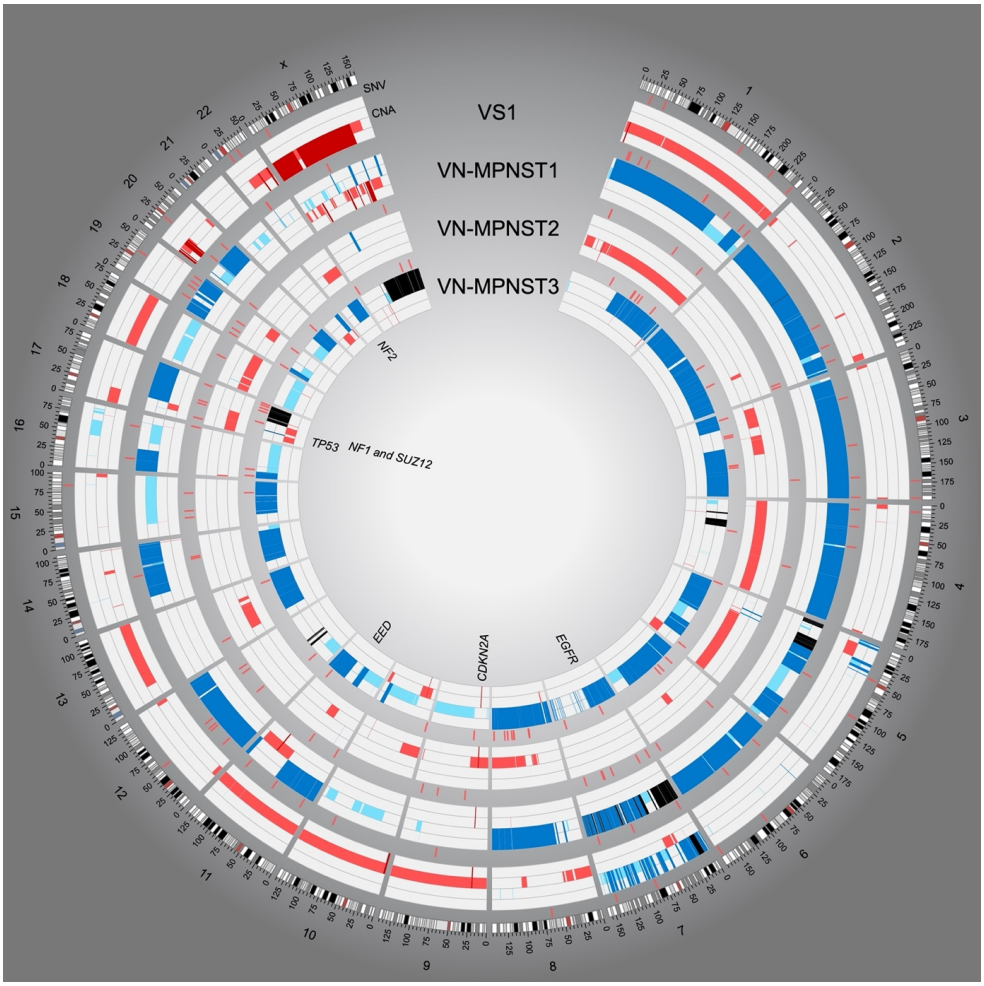

Figure 11. Circos plot of copy number aberrations (CNA) and single nucleotide variants (SNV) in three VN-MPNSTs and one VS. The tracks from outside inwards: chromosome numbers, chromosomal position in Mb, SNV and CNA calls for four consecutive tumors and selected genes previously reported in extracranial MPNST. In the CNA histogram, high level amplifications (CN > 7), high-level gains (CN 4-7) and gain (CN =3) is depicted in black, dark blue and light blue, respectively. Similarly, heterozygous loss and homozygous loss are depicted in light red and dark red, respectively.

was seen in one irradiated and one radiation-naïve tumor. Other notable aberrations previously implicated in extracranial MPNST included high-level gain of *EGFR* (n=2), heterozygous loss of *TP53* (n=2) and heterozygous loss of *NF1* and *SUZ12* at 17q11.2 (n=2). Two tumors, one irradiated and one radiation-naïve, were hyperploid and one

irradiated tumor was hypoploid. Unsupervised hierarchical clustering revealed no association between CNA profile and previous radiation exposure (*Fig.12A*).

Among the small mutations identified, no recurrent mutated gene was identified. Indel/SNV ratio was not associated with prior radiation. Notably, *NF1* was not mutated in any of the tumors. Two tumors, one irradiated and one radiation-naïve, had mutated DNA repair gene. We inferred mutational signatures in VN-MPNST and included 46 sVS from study 1 for comparison (*Fig.12B*). Signature 1, attributed to endogenous deamination of 5-methylcytosine to thymine, was the most prevalent. VN-MPNST formed a subcluster based on contribution of signature 3, a signature associated with *BRCA1/2* aberrations, and all harbored CNA affecting this gene. VS formed two subclusters based on the contribution of signature 6, a signature without known etiology. Notably, unsupervised hierarchical clustering of the signatures did not reveal a cluster associated with previous irradiation in neither 'VN-MPNST nor VS (*Fig.12B and C*).

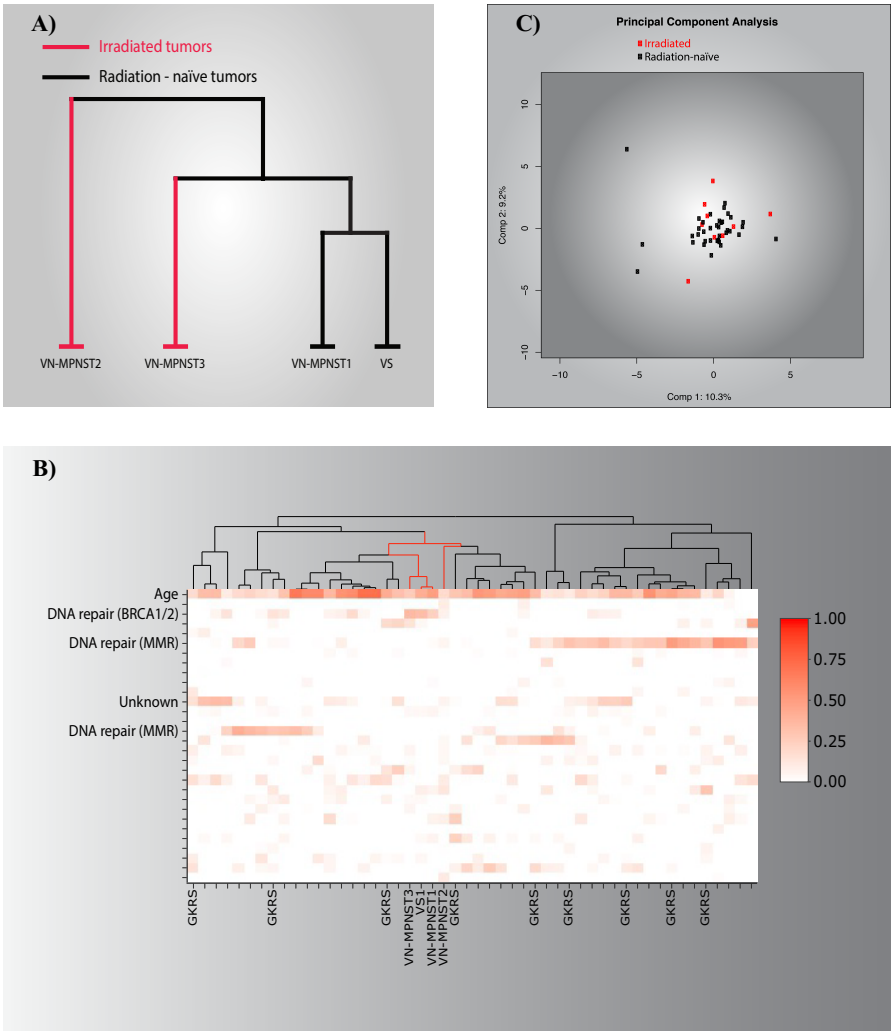

*Figure 12. Unsupervised hierarchical clustering revealed no association between CNA profile and previous radiation exposure (**A**). A matrix depicting the relative contribution of COSMIC mutational signatures in 46 VS, one premalignant VS and 3 VN-MPNST depicted no clustering of the irradiated tumors (**B**). The columns represent the individual tumors with irradiated and malignant tumors marked along the x-axis, whereas the rows represent the 30 different mutational signatures with the signatures contributing the most marked along the y-axis. The results from hierarchical clustering of the mutational signatures are depicted on top of the matrix with malignant tumors highlighted as red lines. Principal component analysis demonstrated no association between radiation and mutational signature (**C**).*

10. Discussion

10.1 Tumor specific mutations in VS

Tumor specific loss-of-function mutations in *NF2* has long been considered a driver mutation in VS and germline pathogenic variants in *NF2* causes the tumor syndrome neurofibromatosis type 2, characterized by bilateral VS. Early studies identified mutated *NF2* in around 70% of tumors and although mRNA levels are comparable to normal controls, most studied tumors demonstrate no merlin protein expression. Substantial evidence, including genomic and functional data, have accumulated over the years and few questions the importance of *NF2* in VS pathogenesis today.[98] Mechanisms for disrupting merlin function, besides intragenic mutations, have also been elucidated, including epigenetic silencing, posttranscriptional regulation and non-coding variants. In study 1, we demonstrated that tumors with wild-type *NF2* harbored mutations in genes that could be linked to *NF2*. Although these results were not functionally validated, they provide a rationale for a mechanism of inactivating merlin function in the absence of *NF2* mutations.

In 2018, Carlson et al. found, for the first time, that all 23 sVS analyzed harbored biallelic somatic mutations in *NF2*.[111] They accomplished this using a combination of WES, RNAseq and a modified WGS approach termed mate-pair sequencing. The mutations identified were intragenic SNVs and indels as well as larger deletions and CNN-ROH of chromosome 22. By compiling the results from our studies 1 and 3, tumors analyzed with both WES, MLPA and DNA microarray have mutated *NF2* in 89% of cases and 44% have biallelic mutations. Our results were similar to that reported in another WES study by Agnihotri et al.[110] As DNA microarray is still considered the gold standard for identifying CNA and CNN-ROH, we do not expect that the addition of WGS, used by Carlson et al., would result in higher sensitivity. Lesions identifiable with WGS, but not with DNA microarray, include translocations and inversions and were not found in VS. One mutation found in the study by Carlson et al. were identified only after manual inspection of RNAseq data, possibly relating to upregulated expression of *NF2* mRNA. This highlights the importance of sequencing

read depth in studies on tumor specific mutations. Recent evidence implicates that VS is a heterogenous tumor, as I will discuss in the next section. Hence, if a small proportion of the cells in a tumor biopsy are neoplastic cells, higher sequencing depth is needed to identify mutations. In most bioinformatic variant call pipelines today, a cutoff at 5% variant allele frequency is used to exclude possible false positives. Hence, with a neoplastic cell content of 10%, a heterozygous mutation will fall on this cutoff limit. Although the different results obtained by us and Carlson et al. could have been explained by sequencing depth, this remains speculation as data on sequencing depth was not provided in the latter study. Another explanation might be the bias towards clinically aggressive tumors in the study by Carlson et al. as these have been shown to more often harbor biallelic mutated *NF2*.[183] A recent study utilizing NGS with a sequencing read depth of 1000x in combination with MLPA, microsatellite analysis and chromosome analyses found mutated *NF2* in 161/188 (86%) tumors.[184] Hence, it seems that the true mutation frequency of *NF2* in VS approximates 90%.

The number of driver mutations needed to initiate and progress neoplastic disease is still controversial. However, data from epidemiologic, genomic and experimental studies suggests that more than one driver event is needed.[48,81] A multi-hit tumorigenesis has also been suggested in schwannomatosis-related VS, where pathogenic germline variants in the tumor suppressor gene *LZTR1* need to be complemented by somatic *NF2* mutations for VS to develop.[185] Hence, with the advent of unbiased genomics approaches, the quest for identifying additional driver mutations in VS has been ongoing for the last few decades. In the first NGS study on schwannoma, a combination of WES and RNAseq were used to assess somatic mutations in 13 VS and 13 spinal schwannomas. In addition to finding *NF2* mutation in 77% of the analyzed tumors, some novel genes were implicated. Most notably, a recurrent *SH3PXD2A-HTRA1* fusion was identified in 12/125 tumors (including a validation cohort). The fusion product was shown to activate the ERK pathway and stimulate tumorigenesis and pharmacological inhibition reversed this. Our group analyzed a total of 121 VS with RT-PCR to identify the fusion in one patient, suggesting that this is a rare event.[186] Other recurrent mutated genes included *ARID1A* (14%), *ARID1B* (18%), *DDR1* (11%) and *TSC1* (9%). In the study by Carlson et al.,

only *NF2* was identified as recurrent mutated gene. However, they identified chromosomal aberrations outside chromosome 22 in clinically aggressive tumors. A linked-read WGS study on 10 familial VS, also found several novel large aberrations including a deletion of *TSPAN8* in the fast-growing tumors.[112] In another recent NGS study on 12 VS, several novel genes mutated in subsets of VS was identified.[113] Previous unbiased approaches to detect CNA and chromosomal aberrations, utilizing DNA microarrays and comparative genomic hybridization, have also detected novel aberrations in subsets of VS in addition to chromosome 22 loss. As summarized in a review by Sass et al., different genes have also been suggested as drivers in VS in studies on RNA and proteins.[109]

Our study 1 is the largest study utilizing NGS to discover tumor specific mutations in VS. We identified a median of 14 likely functional tumor specific mutations. This is a modest number of mutations and mostly in line with similar studies on VS. In addition to *NF2*, we identified *CDC27* and *USP8* as recurrently mutated in subsets of VS. Thus, it seems like all NGS studies finds novel recurrent genes mutated in subsets of VS, but they are apparently rare as the findings are not similar across the different studies. We also identified the axonal guidance pathway as mutated in 25/46 VS. This pathway has been implicated as a driver in pancreatic cancer. Agnihotri et al. also performed pathway analysis on their set of mutations but did not identify the axonal guidance pathway. In study 3 we identified chromosome 22 loss or CNN-ROH as the only significant aberration in agreement with previous studies. Notably we found another 37 recurrent aberrations that were either normal variants or did not encompass coding DNA. It is still possible that some of these aberrations disrupts regulatory regions in the genome and might have a functional consequence in VS. However, to pursue this lead, one would have to analyze a larger cohort to identify potentially tumorigenic aberrations in non-coding DNA.[187] We did not find any recurrent focal CNA, in agreement with Carlson et al., and concluded that this kind of mutation is not likely a driver in VS.

In our opinion, it seems likely that *NF2* disruption is universal in VS and that additional driver mutations are more diverse. Finding these rare drivers in VS, if they exist, has proven hard as results from the different NGS studies do not align. This has

proven a hurdle in the whole cancer genetics field as well where the major drivers for most neoplasms have been identified, but with a lot of minor drivers still missing.[81] The quest for drivers is further complicated by the fact that in apparently normal tissue, clonal selection of known cancer driver genes occurs.[188] Identifying rare driver mutations in VS requires larger sample sizes than currently included in NGS studies. In addition, larger validation cohorts from several research centers should be screened for the newly implied genes in VS. Further, novel mutations need to be functionally characterized to establish them as driver mutations. Indeed, there is a possibility that high-throughput functional characterization of identified tumor specific mutations will become reality soon.

10.2 Intratumoral heterogeneity in VS

The gross appearance of VS is that of a firm rubbery mass intermingled with variable amounts of soft areas. In the microscope it appears with a variable admixture of cellular Antoni A areas and hypocellular microcystic Antoni B areas.[189] Some authors have suggested that the Antoni B areas represents degenerated Antoni A areas with immune cell infiltrates.[15] Small amounts of normal tissue, including endothelial cells and infiltrating immune cells, are generally acknowledged on histopathological examination, but the tumor has traditionally been assumed to consist of mostly neoplastic cells. However, immunohistochemical studies of VS has elucidated that immune cell infiltration and intratumoral hemorrhage, rather than neoplastic cell proliferation, accounts for tumor growth.[10] A follow-up demonstrated that the immune cell infiltrate is made up of mostly tumor-associated macrophages of the M2 type.[11] These findings were recently replicated in an in vivo study, estimating that as much as 70% of the cell content in growing VS is macrophages.[13] A recent proteomic study also found markedly intratumoral heterogeneity not acknowledged in standard histopathological examination.[190] Our data on tumor specific variants also indicated the presence of intratumoral heterogeneity. In study 1, *NF2* variant allelic frequencies ranged from 8% - 69% and in study 3 we estimated that the chromosome 22 aberrated tumors had a neoplastic cell content ranging from 25% - 94%. Since the amount of

immune cell infiltration in VS is correlated with growth, we hypothesized that aberrant cell fraction, which we assume to be inversely correlated with the fraction of immune cells, should be inversely correlated with volumetric growth. We did not find any such correlation. However, we could only calculate volumetric growth in 16/55 (29%) tumors due to missing serial preoperative MRI in the rest of the cases. ASCAT estimates aberrant cell fraction based on the presence of large aberrations. Hence, aberrant cell fraction could not be estimated in tumors without chromosomal aberrations. However, based on allelic frequencies of both small and large aberrations found in this work, it is likely that most or all VS are genetically heterogenous. Further, based on the previous works mentioned here, it appears that tumor-associated macrophages make up a substantial portion of the cells in VS (*Fig.13*). We did not find different CNAs with different allelic frequencies within the same tumor and hence, we did not find any evidence of different neoplastic clones within any VS.

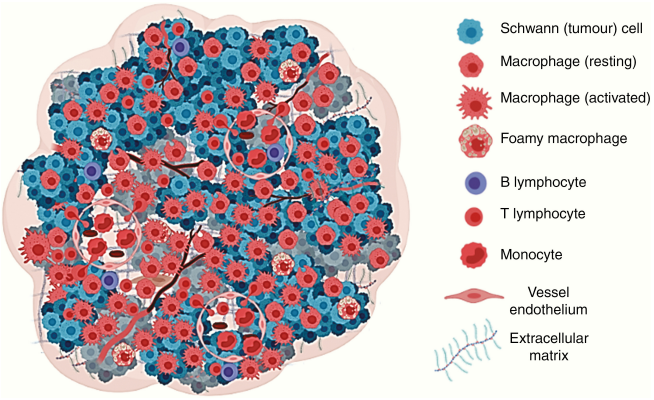

Figure 13. Intratumoral heterogeneity in VS as it is depicted by Hannan et al.[1]

10.3 Gamma Knife Radiosurgery

Two questions relate to genetics in GKRS treated VS: 1) Does IR leave a footprint in the VS genome and 2) is there any genetic biomarker of radioresistance?

10.3.1 IR-induced malignancy

VS is a benign tumor that in general does not become malignant. However, a VS can undergo malignant transformation, albeit it is a very rare event. There has been

reports demonstrating malignant transformation of a previously benign VS both in the presence and in the absence of SRS.[125] Hence, it is important to clarify the role of SRS in the transformation. For VS receiving GKRS as the initial treatment, Cahan's criterion 2 is difficult to adhere to as these are not verified histologically.[116] However, contrast-enhanced T1-weighted MRI is considered the gold standard for diagnosing VS and hence, serve as a viable surrogate for histological examination. Also, a typical latency period beyond 5 years between primary diagnosis and subsequent malignancy lends support to the notion that the tumor was not malignant from the start. Hence, the main question is whether the malignant transformation was SRS induced or spontaneous. Although several epidemiological studies exist on the subject, indicating no higher incidence in SRS treated patients than in the population, few genetic studies have been undertaken.

As discussed in the introduction, IR might leave a unique mutational fingerprint in an irradiated tissue. An increase in mutations after SRS might increase the chance of inducing cancer driver mutations and induction of genomic instability will certainly increase the chance of malignancy. In our studies 1 and 3 however, we found no mutational signature based on neither small mutations nor CNAs that were associated with radiation exposure. Aaron et al. identified 23 and 184 tumor specific mutations in two irradiated tumors as compared to an average of 26.5 mutations in radiation-naïve tumors.[113] Based on this, they concluded that SRS induces a hypermutator phenotype. In our study 1 we also identified one tumor with 231 mutations, as compared to an average of 16 across the cohort. However, this was a radiation-naïve tumor carrying a mutation in the DNA repair gene *RAD54L*. The irradiated tumors harbored no more mutations than the radiation-naïve. Hence, the higher mutational load in these tumors is probably better explained by other factors than exposure to IR. Aaron et al. also stated that irradiated tumors seemed to have a higher burden of CNA, although they conclude that the genomes are fairly diploid, which is also evident from the data presented. In our study 3 on CNA in 18 irradiated and 37 radiation-naïve VS, number of CNAs were similar between the two groups. The radiation-associated signature found by Behjati et al. consisted of an excess of small deletions (sized 1-100 bp) and balanced inversions.[138] With WES we were able to characterize small deletions in the

exome of VS and found that there was no increase of deletions in irradiated tumors. However, WES captures only around 2% of the genome leaving it less powered to detect genome-wide patterns. The DNA microarray used in study 3 is designed to detect aberrations with a resolution of 25-50 kbp and upwards and therefore is unable to detect these small deletions. Neither WES nor DNA microarray can detect balanced inversions. Hence, to detect the novel IR signature described by Behjati et al., one needs to analyze VS with WGS.

We had the opportunity in study 4 to assess the genome of malignant transformed VS exposed (n=2) or unexposed (n=1) to GKRS. No single locus or genome-wide pattern could be associated with radiation. In fact, one radiation-naïve and one irradiated tumor clustered together based on mutational signatures and they both demonstrated genome-wide hyperploidy and chromothripsis of chromosome 7. The latter finding contrasts with the findings by Youk et al. where chromothripsis was found to be associated with IR.[134] Our conclusions are obviously limited by the small sample size.

An important implication from the study by Youk et al., is that apparently normal cells residing in the field of radiation, acquires numerous mutations as a direct consequence of the radiotherapy.[134] 1 Gy of IR was estimated to induce 16 mutations with a preponderance of small deletions and structural variants. Somatic mutation rate for SNVs in normal tissue has recently been measured to 2.66×10^{-9} per base per mitosis, or approximately 10 SNVs per mitosis in any given somatic cell. A measurement of somatic mutation rate of indels and CNAs has not been done yet. However, germline data suggest they are much rarer. A large population study estimated that an individual's genome differed from the reference genome at 4-5 million sites, with >99.9% being SNVs and small indels.[191] This provides an estimate on the relative distribution of mutation rate in germline. Hence it is clear that IR will substantially increase the mutational load in the irradiated tissue. The IR-induced mutations affect the genome at random and may, hence, induce oncogenic driver mutations.

To detect an IR footprint in the genome of VS using bulk analysis, IR must have induced mutations giving the harboring cell a selective growth advantage over

neighboring cells. Given common variant calling practice, a cutoff at 5% variant allele frequency will exclude IF signatures present in individual cells. Because IR affects the genome at random, every single cell in the irradiated field will have a unique mutational signature. And this signature must be positively selected to be detected. It can be argued that it also needs to be positively selected for it even to be clinically relevant. However, the random generation of new mutations will always confer a risk, even if very small, of inducing malignant transformation. We believe IR will also induce mutations in the irradiated tissue in and surrounding a VS and might therefore increase the risk of malignant transformation. However, in line with both our papers, and the existing epidemiological data, the risk is very small.

10.3.2 Radiosensitivity of VS

As outlined in the introduction, IR both directly damages DNA and produce reactive oxygen species in VS. However, viable cells exist even after large doses of radiation.[139] These observations are often attributed to the low proliferative capacity of VS cells. This allows dormant VS cells in interphase enough time to repair DNA damage before it enters cell cycle. The demonstration of upregulated DNA repair after exposure to IR as well as pharmacological inhibition of DNA repair resulting in increased radiosensitivity supports this theory.[141,142] Loss-of-function mutations in any of the genes responsible for DNA repair would certainly impair this ability. However, according to our study, this is rare in VS. Radioresistance was not associated with mutated DNA repair genes in neither benign nor malignant VS. Lee et al. found that irradiated VS were diploid at chromosome 22 in contrast to radiation-naïve VS.[140] However, in our study of a larger cohort of irradiated tumors we saw no such difference. The existing theories on the cause of radioresistant VS can be summarized as follows:

1) Low proliferative capacity
2) Enhanced DNA repair
3) Increased ability to mitigate effects of reactive oxygen and nitrogen species
4) Increased angiogenesis after exposure to IR

Evidence for theory 1 was given by Hansen et al. who demonstrated that the number of apoptotic cells following IR was proportional to the number of proliferating cells.[141] The fact that rapidly dividing cells are more radiosensitive is common knowledge in radiobiology. According to our studies, we believe that enhanced DNA repair is not an important mechanism of radioresistance in VS. This is also supported by the fact that repeat SRS is effective in most patients failing first SRS.[192] If there was a genetic determinant of radiosensitivity, one would not expect that SRS resulted in different outcome in the same patient. However, if timing of SRS with cell cycle status is the determining factor of radiosensitivity, the outcome could differ in the same patient at different time points. I.e., if cells in the treated tumor are actively replicating at the time of SRS, the tumor would be more radiosensitive than if the cells are dormant. To harness this clinically, one would need a non-invasive method, e.g., positron emission tomography (PET) with a proliferation tracer, to measure the number of replicating cells in a VS to determine when SRS is appropriate.[193] Further, it has been demonstrated that IR induces oxidative stress in VS, but upregulated c-Jun N-terminal kinase activity counteracts its consequences.[146,147] Oxidative stress is known to induce mutations, and hence, this is consistent with our findings that irradiated VS do not harbor more mutations than radiation-naïve VS. However, it is unclear whether this is a feature of VS in general or for radioresistant VS specifically. Regarding theory 4, there is a possibility that the variable rate of radiosensitivity in VS relates to tumor associated endothelial cells rather than neoplastic cells.[144,145] Indeed, endothelial cells have been shown in functional studies to be highly radiosensitive.[194,195] However, since immunohistochemistry staining of *VEGFR* was not done before and after SRS in the same tumor, it is not known whether increased angiogenesis is an inherent capability of the VS or the result of IR.

10.4 Etiology

A viral etiology has been established in approximately 10% of all cancers worldwide.[196] Viruses are also responsible for a wide range of benign neoplasms.[197] A

suspicion of viral etiology might arise from several aspects and observations of the neoplastic disease in question: [198]

1) A specific geographical distribution as seen in the case of liver cancer, caused by hepatitis virus B and C.
2) Distinct clinical features as was seen with cervical cancer appearing almost exclusively in sexually active women.
3) Increased risk of a neoplastic disease in immunocompromised individuals as was seen with Merkel cell carcinoma in patients with AIDS.

The peculiar preponderance of intracranial schwannomas being located at the vestibulocochlear nerve rises the suspicion of an exogenous factor. Viruses are known to infect both the inner ear and the vestibular nerve in animal models.[199] Also, viruses cause measles, mumps and presumably vestibular neuritis and may establish latency at the origin of the VS. Two previous studies have investigated the possibility of a viral etiology in VS with negative findings.[40,41] However, they were both biased towards herpesviruses. Our study represents, to our knowledge, the first unbiased search for viral nucleic acids in VS. Utilizing different unbiased complementary approaches, we did not find any plausible viral association with VS. We did find HERVK transcripts, but they were both up- and downregulated compared to normal nerves. HERVK belongs to a family of endogenous retroviruses that became incorporated into the human genome >500´000 years ago and its role in human disease is debatable.[200] HERVK transcripts have been found in a wide range of cancers and might be a prognostic factor.[201] Altered HERVK transcript levels in VS might represent a lack of regulation and we do not believe they are causative in VS. It seems unlikely that VS is caused by a virus, although novel viruses, a “hit-and-run” mechanism or viral epigenetic reprogramming cannot be excluded by our study.[202-204]

As outlined in the introduction, several possible etiologies have been explored regarding developing VS without producing compelling evidence, including our study on viral nucleic acids in VS biopsies. It is possible that endogenous mutational rate in normal cells is sufficient to provide the substrate for which cells acquire a selective growth advantage and ultimately a neoplasm.[205,206] Cancer risk in a given tissue has even been shown to correlate with the rate of replication in its corresponding normal,

non-cancerous, stem-cells, providing evidence for the “bad luck” theory in cancer etiology.[207] A study demonstrating brain volume as a risk factor for glioblastoma, also support this line of thinking.[208] Hence, there is no need for an exogenous carcinogen explaining the development of a VS. However, the preponderance of intracranial schwannomas being located to CN8 is still not understood. Both intracranial and spinal schwannomas have a predilection for sensory nerve root ganglia, pointing to an inherent vulnerability for merlin disruption in these cells.[209] Tryggvason et al. even suggested that satellite glia cells, the main glia cells in sensory nerve root ganglia, are the founding cells of schwannomas. Satellite glia cells are thought to represent developmentally arrested Schwann cells and share many histologic features, making it hard to distinguish the two.[210] Both satellite glia cells and dedifferentiated Schwann cells express the stem cell marker Sox2.[211,212] This points to the intriguing possibility that satellite glia cells, retaining a more stem cell-like phenotype, is dependent on merlin for growth inhibition and that upon its disruption, forms a schwannoma.[213,214] This could explain why most schwannomas arise from sensory nerves and warrants further studies.

10.5 VN-MPNST

For the first time, we have analyzed the genome of a VS undergoing malignant transformation. Most strikingly were the findings in the VS with large aberrations, homozygous deletion of *CDKN2A* and CTLP, in stark contrast with our studies 1 and 3 on VS genetics. However, biallelic loss of *NF2* was similar, suggesting it was in fact a VS. The fact that the nonsense *NF2* mutation was not detected in the tumor after malignant transformation might indicate the coexistence of two different tumor clones. However, the genomic CNA pattern was similar, contradicting this. The precancerous VS demonstrated evidence of genomic instability with a near whole-genome doubling and acquisition of mutation in three known cancer genes completing the transformation. The precancerous VS demonstrated a high contribution of mutational signature 3 associated with aberrant *BRCA1/BRCA2* functioning and failure of DNA double-strand breakage repair. This signature did not contribute significantly to any of

the VSs from study 1 but was prevalent in all VN-MPNSTs. Our study indicates that there exist a precancerous VS in a continuum from VS to VN-MPNST. A basic genetic test of the VS biopsy, e.g., with a Q-PCR assay detecting the deletion of *CDKN2A*, would be able to diagnose this patient with a precancerous tumor. This would have guided the treating physicians to gross total resection instead of the facial nerve preserving strategy commonly employed in VS surgery. Although this patient would benefit from such an approach, the rarity of malignant transformation in VS suggests that it would not be cost-effective for VS patients in general. However, it could be considered if any clinical or pathological characteristics raise suspicion.

VN-MPNST is exceedingly rare with a recent study estimating that for every 1041 VS, one VN-MPNST will be diagnosed.[154] With approximately 100 VS diagnosed in Norway every year, this equates around one VN-MPNST every 10 years. Hence, establishing evidence-based treatment for these patients is hard and mostly relies on case reports. In our study 4, we found that VN-MPNST shares genomic features with its extracranial counterpart. In particular the predominance of large over small mutations, grossly aberrated genomes and homozygous deletion of the tumor suppressor *CDKN2A* is similar (Fig.14). Although small mutations in *NF1* and members of the Polycomb repressive complex 2, commonly seen in extracranial MPNST, was not seen in our study, CNAs affecting the genomic loci of these genes were seen in all tumors. Hence, we believe that intracranial and extracranial MPNST share a common pathogenesis and that clinical trial results from extracranial MPNST might be extrapolated to include intracranial MPNST.

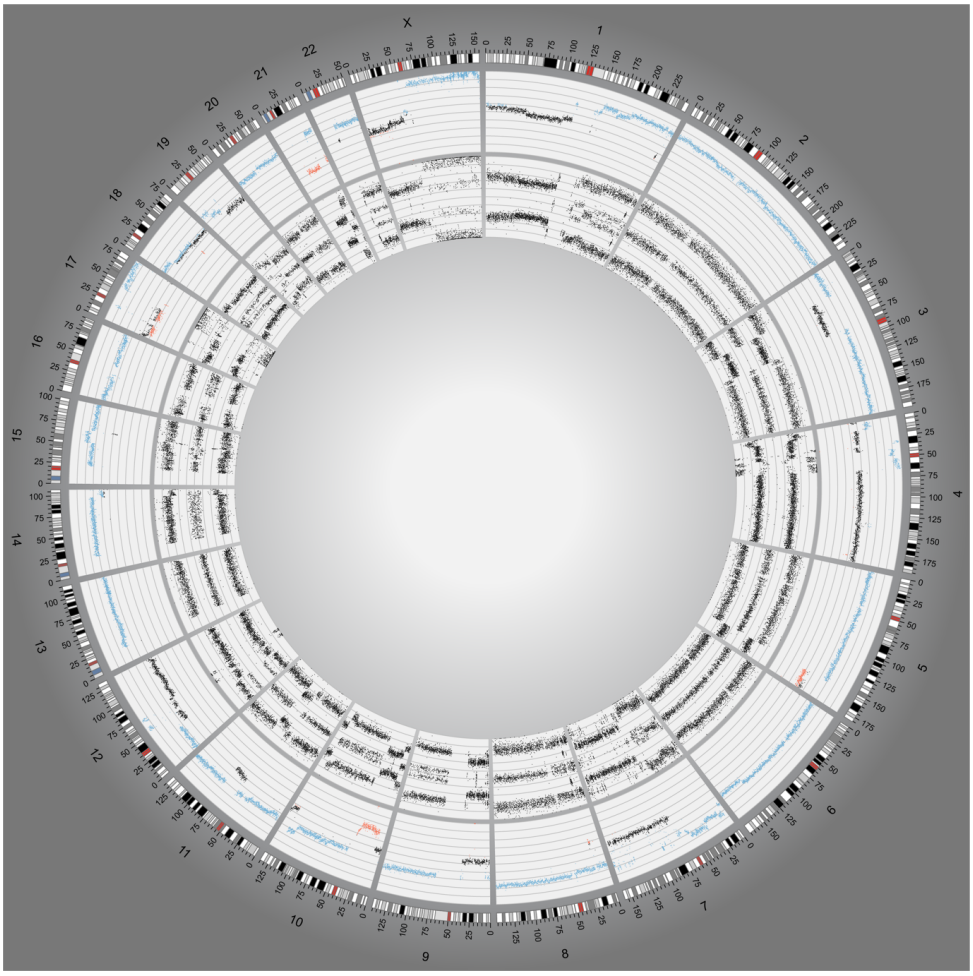

Figure 14. Circos plot of a VN-MPNST karyogram demonstrating hyperploidy and chromothripsis at chromosome 7.

10.6 Limitations

10.6.1 General considerations

One limitation applicable to most basic science conducted on VS is the use of bulk analysis on a heterogenous cell population. As most cancers are heterogenous, possibly consisting of different tumor cell clones and an admixture of normal cells,

constituting the tumor microenvironment, bulk sequencing analysis picks up mutations from all these cooccurring clones.[215] Possible pitfalls in bulk analysis are false positives and false negatives. False positives might arise if observed changes in DNA, RNA, protein or other biological characteristics are inferred to represent neoplastic cell aberrations initiating or progressing the tumor when they actually stem from infiltrating normal cells. In DNA studies, typically using leukocyte DNA as normal control, this risk is minimized as the variants existing in infiltrating immune cells are filtered out. Remaining stromal cells make up a much smaller proportion of cells in VS and would probably not preclude the analysis. However, in studies of RNA, which typically uses normal nerves as control, the changes in expression observed in the tumor might very well stem from normal cells. False negatives might arise if the normal cell population make up such a large proportion of the tumor that a typical DNA variant do not reach the common filtering threshold of 5%. In VS, it has been shown that macrophages might constitute 70% of the tumor.[13] If the sequenced biopsy also contains a significant proportion of stromal cells, it will leave a small proportion of neoplastic cells with the risk of missing true variants. Although there is no evidence for it in VS, a mixture of different neoplastic cell clones would further lower the fraction of cells harboring a specific mutation.

Another limitation applicable to all basic VS research, is the inherent characteristics of the study population. Given the current treatment guidelines, small and indolent VS is followed with observation or treated with SRS. Today, only around 30% of VS patients undergo surgery. Tissue biopsy for diagnostic purposes is not done as it currently has no consequences for the management. Hence, adhering to Hippocrates' principle of *primum non nocere*, the tissue available for analysis is highly biased towards large, growing and/or radioresistant VS. Knowledge gathered from basic research on VS might therefore not be representable of most patients. Even so, the translational potential of basic research on VS is covered as the biological processes underlying the need for surgery is the most pressing clinical issue in the management of VS patients.

Throughout this project, we have had a special emphasis on identifying specific genetic features of irradiated VS and VN-MPNST. Although our study design is well

suited to characterize most exonic mutations, the genomic characterization is limited to mutational events leading to shift in gene dosage and/or allelic imbalance, i.e. CNAs and/or CNN-ROH. Since a genomic signature of balanced inversions has been associated with IR exposure, we think that assessment of balanced mutational events in irradiated VS is warranted.

10.6.2 Specific limitations

Study 1

In this study we used matched leukocyte DNA as normal control to minimize the adherent limitations associated with bulk analysis addressed above. In addition, there are some issues that needs to be addressed regarding the WES design. Prior to sequencing, oligonucleotide probes are used to capture a target region consisting of most of the coding DNA as well as part of the regulatory DNA, totaling 64 Mbp (approximately 2% of the genome). Although most driver mutations in cancer arise in coding DNA, some tumors also harbor driver events in non-coding DNA and those could be missed in our study.[187] Further, uneven coverage is a common problem with exome capture kits that might result in missing true variants.[216] Although we achieved an average exome coverage of 91x in our study, 5% of the target regions had coverage below 20x. Our study represents the largest NGS study on VS to date, and we believe it is well powered to identify common driver events. However, as is evident from the divergent results from the NGS studies published, one might miss the rare driver events operating in a subset of VS patients. WES of tumor-normal pairs is a powerful tool for detecting tumor-specific SNV and indel, but is not well suited to detect CNAs, mainly due to uneven coverage. We addressed this issue by utilizing DNA microarray in study 3.

Study 2

In this study, tumor RNA was analyzed with the ViroChip microarray and RNAseq and compared to normal nerve RNA. As discussed above, this raises the issue of false positives in bulk analysis. However, as we approached a discovery design, false negatives would pose a greater obstacle. Indeed, the ViroChip yielded several false

positives which were excluded in follow-up analyses. Several features of the ViroChip probably contributed to these results, including non-specific probe hybridization, the use of stool samples for normalization of probe signals and specific signatures present in VS as compared to tibial nerves. It seems like the ViroChip has high sensitivity and low specificity, which is acceptable in a discovery study. The follow-up analyses we performed, PCR based techniques, RNAseq and WGS, are highly specific and was able to rule out most of the findings from the ViroChip. Although we believe our study design has a high sensitivity for detecting viral sequences in VS, novel viruses without sequence homology to known viruses, viral "hit-and-run" mechanisms and viral epigenetic reprogramming cannot be excluded.

Study 3

DNA microarray is more prone to false negatives caused by infiltrating normal cells than are WES. To call a CNA from microarray data, one is dependent on a shift in the intensity values of copy number probes and SNP probes over a stretch of at least 25-50 kbp. Theoretically, a heterozygous loss should result in homozygosity, evidenced by BAF values of 0 or 1, and a 50% decrease in the copy number probe values. With increasing amounts of normal cells, these values will be shifted towards a normal diploid pattern. In our study we utilized ASCAT to mitigate these effects and were able to call large chromosome 22q deletions with an aberrant cell fraction as low as 20%. We did not identify any recurrent focal CNA in our cohort and this might be due to detection issues. However, it is in agreement with another study utilizing WGS to study CNAs in VS.[111] We did not use matched normal control for the DNA microarray, but relied on a panel of normal samples. The chance of missing a driver variant with this approach is small, as we expect that normal variants are not tumorigenic. We expect that several of the CNAs called in the tumors in fact represent germline variants. To exclude these variants, we utilized GISTIC to identify significantly aberrated regions across the cohort. These variants were further filtered against databases of normal variants and according to genomic annotation. In fact, out of 38 identified regions, only the chromosome 22q deletion remained after filtering. As mentioned earlier, it is possible that we excluded true driver variants in non-coding

DNA, although it would pose a greater challenge to infer these in VS tumorigenesis. We also included WES data to expand our study cohort. WES data are less suitable at detecting focal CNAs but using DNA microarray data as a training data set, we were able to call large aberrations in the WES data. DNA microarray is dependent on a shift in gene dosage and/or allelic imbalance to call a variant, and hence, will not detect mutational events like balanced inversions and translocations. An unbiased assessment of such mutations would require a WGS approach.

Study 4

The limitations associated with the technologies used in this study have already been discussed above. An additional limitation is the small sample size of 3 VN-MPNSTs, including 2 irradiated. It is therefore possible that our samples do not represent this entity in general. However, the genomes analyzed in our study shares many features with extracranial MPNST, adding external validation. Although our study design is well suited to finding driver events in these tumors, it is less suitable at characterizing genomic mutational signatures. The signatures presented in this paper must be considered in the light of the exome design, covering 2% of the genome. Mutational signatures might elucidate the etiology and hence, we believe WGS is warranted to further assess this issue.

10.7 Future perspectives

This thesis provides the to date most comprehensive unbiased assessment of genetic aberrations in VS, VN-MPNST and irradiated VS. Together with four similar studies utilizing NGS, the genomic landscape of VS is being elucidated. The main driver of tumorigenesis is disruption of *NF2*, confirmed in all studies. However, several other driver events are being described in subsets of VS patients. We believe that as the number of sequenced VS exomes and genomes accumulate, even more rare driver events will be discovered. The case for VN-MPNST is quite different, as only our three exomes have been published so far. Hence, we urge other research groups to collect specimens and sequence to provide a deeper understanding of the genetic aberrations

underlying this disease. As with the cancer genomic field in general, the main limitation hampering clinical translation of these discovery studies, is functional characterization of novel driver events and developing targeted treatments. Our work provides the rationale for further functional characterization of *USP8, CDC27* and the axonal guidance pathway in VS tumorigenesis and *CDKN2A, FOXO4, PDGFRB* and *GNAQ* in VN-MPNST tumorigenesis.

Through our work with irradiated VS, it appears there are no genetic biomarker of radioresistance. Hence, the most likely explanation of the variable radiosensitivity in VS seems to depend on timing with replication. However, few irradiated VS have been sequenced and genetic clues to radioresistance might yet be discovered. Regarding mutational signature, possibly providing a telltale of the genomic effect of IR, a WGS approach should be considered to conclude on the matter. A single-cell WGS approach as was done by Youk et al. would probably be the most sensitive to detect any IR related signature in irradiated VS.[134] In our studies, we did not find any clonally selected IR induced aberrations, which would be the clinically relevant adverse outcome. Hence, this work provides evidence that might be used in counseling of VS patients regarding choice of treatment.

Through our work and the work of others, it has become clear that VS exhibit intratumoral heterogeneity. Evidence points to infiltrating macrophages making up a large proportion of VS and even causes growth. Further work should be carried out to characterize the inflammatory and stromal microenvironment in VS and its role in initiating and progressing the tumor. To conclude on the matter of the composition of cells in VS, either single-cell sequencing or transcriptome sequencing with spatial resolution should be considered.

11. Conclusions

Study 1

In this study we have elucidated the exomes of irradiated and radiation-naïve VS. Disrupted *NF2* is seemingly a requisite for initiating VS tumorigenesis, but probably not enough. We identified several novel alterations in VS, including mutated *CDC27* and *USP8* and clustering of mutations in the axonal guidance pathway. Notably, the irradiated tumors were similar to radiation-naïve tumors. One hypermutated and clinically aggressive VS was found to harbor mutation in *RAD54L,* a DNA repair gene.

Study 2

We utilized several complementary unbiased analyses to conclude that no viral nucleic acid could be associated with VS.

Study 3

In this study we assessed large aberrations, CNAs, in irradiated and radiation-naïve VS to find that deletion or CNN-ROH of chromosome 22q, the locus of *NF2*, is the only recurrent event. Neither genomic copy number profile, nor specific CNAs, correlated with previous exposure to IR. Notably, the tumor cell fraction was estimated to 25-94% indicating intratumor heterogeneity.

Study 4

In this study we demonstrated that there exist a premalignant VS characterized by large CNAs, homozygous loss of *CDKN2A*, chromothripsis of chromosome 7 and biallelic *NF2* inactivation. Whole-genome doubling and mutations in three known cancer genes, *FOXO4, GNAQ* and *PDGFRB*, were responsible for malignant transformation. Further, we showed that the genomes of VN-MPNST is characterized by gross chromosomal aberrations and homozygous loss of *CDKN2A*. No genetic feature was associated with previous exposure to IR.

12. References

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Paper III

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LABORATORY INVESTIGATION

Gamma Knife Radiosurgery does not alter the copy number aberration profile in sporadic vestibular schwannoma

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Abstract

Introduction Ionizing radiation is a known etiologic factor in tumorigenesis and its role in inducing malignancy in the treatment of vestibular schwannoma has been debated. The purpose of this study was to identify a copy number aberration (CNA) profile or specific CNAs associated with radiation exposure which could either implicate an increased risk of malignancy or elucidate a mechanism of treatment resistance.

Methods 55 sporadic VS, including 18 treated with Gamma Knife Radiosurgery (GKRS), were subjected to DNA whole-genome microarray and/or whole-exome sequencing. CNAs were called and statistical tests were performed to identify any association with radiation exposure. Hierarchical clustering was used to identify CNA profiles associated with radiation exposure.

Results A median of 7 (0–58) CNAs were identified across the 55 VS. Chromosome 22 aberration was the only recurrent event. A median aberrant cell fraction of 0.59 (0.25–0.94) was observed, indicating several genetic clones in VS. No CNA or CNA profile was associated with GKRS.

Conclusion GKRS is not associated with an increase in CNAs or alteration of the CNA profile in VS, lending support to its low risk. This also implies that there is no major issue with GKRS treatment failure being due to CNAs. In agreement with previous studies, chromosome 22 aberration is the only recurrent CNA. VS consist of several genetic clones, addressing the need for further studies on the composition of cells in this tumor.

Keywords Vestibular schwannoma · Gamma Knife Radiosurgery · Whole genome microarray · Intratumor genetic heterogeneity · Neurosurgery · Genetics

Introduction

Vestibular schwannoma (VS) is a benign intracranial neoplasm originating from the Schwann cells surrounding the vestibular portion of the 8th cranial nerve. VS makes up 8% of intracranial tumors, with an annual incidence rate ranging from 10 to 22 per million [1, 2]. Although patients have a normal life expectancy, they experience significantly reduced quality of life attributable to dizziness, headache, hearing loss, facial nerve palsy and tinnitus [3]. Inactivation of the *NF2* tumor suppressor gene is considered an initiating event in VS tumorigenesis, but it is likely that other factors also contribute [4, 5]. During recent years, several novel genetic events have been linked to the disease [6–8].

Gamma Knife Radiosurgery (GKRS) is a type of ionizing radiation therapy commonly used to treat VS. There are controversies regarding whether ionizing radiation might induce malignant degeneration or second neoplasms [9, 10].

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The risk for inducing neoplasms following ionizing radiation demonstrates a linear dose-response relationship, thus making it theoretically feasible for GKRS inducing neoplasms in the normal tissue surrounding VS [11].

The aim of this study was to analyze the genome of 55 sporadic VSs (sVS) to assess whether GKRS induce copy number aberrations (CNA). To understand the mechanism, we wanted to evaluate whether there are any genetic aberrations associated with GKRS treatment resistance. Previous studies on structural changes in the VS genome have identified chromosome 22q loss as the only recurring event, present in 25–83% of VSs [12]. However, previous studies have used techniques with lower resolution. Hence, our secondary aim was to characterize CNAs at a more detailed level as well as using this data to evaluate intratumor heterogeneity.

Materials and methods

Patient samples

VS tissue and matched blood sample was collected from 55 patients without a history of NF2, who underwent first-time suboccipital resection of unilateral VS at the Department of Neurosurgery, Haukeland University Hospital, from August 2003 to May 2017. Eighteen patients had been previously treated with GKRS for the same VS. Written informed consent was received from all patients before tissue harvesting and the study was approved by the Regional Ethical Committee for medical research in Western Norway (2013/374). Tumor samples were harvested from the subcapsular part and snap frozen and stored in liquid nitrogen in the Bergen Neurosurgical Tissue Bank at Haukeland University Hospital. All samples underwent routine histology. Volumetric tumor measurements were performed on BrainLab Elements if preoperative MRI scans were available (Version 2.4.0, BrainLab AG, Munich, Germany).

DNA extraction

DNA was extracted by disrupting the tumor tissue with the TissueLyser (Qiagen, Hilden, Germany) followed by protease treatment. DNA was then extracted using the QIAamp DNA Mini Kit (Qiagen). The DNA quality and quantity were evaluated with 1% SeaKem gel electrophoresis and NanoDrop (Thermo Fisher Scientific), respectively.

Whole-genome DNA microarray

The CytoScan HD microarray (Affymetrix, UK) was used to detect chromosomal aberrations according to the manufacturer´s recommendations. CNAs were called using three different software: (1) chromosome analysis suite v3.2 (ChAS, Affymetrix, UK), (2) Rawcopy [13] and (3) Nexus Copy Number (BioDiscovery, El Segundo, CA, USA). All data were mapped to the hg 19 reference genome build. We applied the following filtering criteria for including the called CNAs in downstream analysis: (1) marker count ≥ 90 for gains; (2) marker count ≥ 30 for losses; (3) visual confirmation for mosaic variants; (4) segment size ≥ 1 Mbp for copy number neutral runs of homozygosity (CNN-ROH). Recurrent CNN-ROHs were further inspected for harboring small variants in whole-exome sequencing (WES) data using IGV[14]. BEDTools was used to produce a per sample union CNA call set, merge fragmented calls and to identify common regions harboring CNA across the cohort [15]. Candidate CNAs were manually inspected in IGV and filtered based on the following criteria: (1) variant not present in databases of copy number variants (CNV) in normal healthy controls (Affymetrix reference database with $n = 2691$, Database of genomic variants as per May 2016 [16]); (2) variant containing NCBI reference sequence gene; (3) variant present in 3 or more samples. GISTIC [17] was used to identify statistically significant aberrated regions across the cohort.

For estimating aberrant cell fraction and allele specific copy number profiles in the tumors, the Allele-Specific Copy number Analysis of Tumors 2.5.2 (ASCAT) software was used [18]. Per sample log ratio (LR) and B-allele frequency (BAF) values from the 27 tumors analyzed with Rawcopy was used for input. ASCAT was run with default parameters except from gamma which was set to 0.45 in compliance with the estimated compression factor in the Affymetrix CytoScan HD microarray.

Whole-exome sequencing (WES)

WES data were available from a previous study on 46 sVSs, including 18 samples also analyzed with microarray [7]. The Sequenza software version 2.1.2 was used for estimating aberrant cell fraction and calling allele-specific copy number profiles from the BAM files [19]. The 18 samples analyzed with both microarray and WES were used as training data to set the following parameters for running Sequenza: gamma = 100, kmin = 30 and median normalization method. Aberrant cell fraction estimates below 0.20 were not included as the software was not trained to estimate at this level.

Statistical analyses

Statistical analyses, including descriptive statistics, contingency table statistics, Mann-Whitney U test and linear correlation, were done using Nexus Copy Number and/or R [20]. Clustering of the sample set based on CNA profiles was done with Rawcopy using the hclust R package as well

as with the built-in complete linkage hierarchical clustering algorithm in Nexus Copy Number.

Results

Patient characteristics

55 patients presenting with sVS were included (Table 1). Mean age at the time of surgery was 53.3 years ranging from 18 to 80 years. Mean preoperative tumor volume was 8.4 cm^3 ranging from 0.37 to 26.78 cm^3. 18 patients underwent Gamma Knife Radiosurgery (GKRS) prior to surgical removal of the primary tumor. Mean time between GKRS and surgery was 1429 days ranging from 280 to 3478 days, and the margin dose in all cases was 12 Gy. All but three GKRS treated patients needed surgical removal because of post-treatment growth; VS14 experienced dizziness, VS16 acquired an intratumoral cyst and VS26 developed trigeminal neuralgia. Five patients had cystic tumors.

Chromosome 22 aberration is the only recurrent copy number aberration in sVS

Using the union call set from filtered ChAS and Rawcopy segments, a median of 7 (0–58) CNAs per sample was identified. Figure 1 illustrates the karyogram of a representative sVS. A median of 0.17% of the sVS autosome was affected by CNA. 38 genomic loci were found to harbor a CNA in three or more samples. However, all but the chromosome 22 loss were common variants (CNV) present in healthy controls. A median of 3 (0–134) CNN-ROHs were seen in the tumors. None of the recurrent regions across the samples harbored any point mutations or indels. ASCAT was then used to infer aberrant cell fraction and absolute allele specific copy number. The number of CNAs identified by the different approaches were highly correlated ($r = 0.831$, $p < 0.001$). ASCAT identified a median of 21 (2–219) autosomal CNAs with a median gain-to-loss ratio of 1.25 (0.25–6.50). The only recurrent CNA retained after filtering was chromosome 22 loss or CNN-ROH. GISTIC analyses on segmented data from Rawcopy and Nexus Copy Number identified chromosome 22 loss as a significantly recurrent event (Q-bound $= 1.36 \times 10^{-9}$, G-score = 15.72). Other events identified were either CNVs or non-coding DNA. Neither tumor volume, volumetric growth nor age was significantly associated with chromosome 22 status, number of CNAs or aberrant cell fraction. Aggregating the results from ASCAT and Sequenza, 25 out of 55 (45%) tumors harbored a chromosomal aberration at chromosome 22 including seven tumors with CNN-ROH, 17 tumors with loss and one tumor with a loss followed by a CNN-ROH (Table 2). Most aberrations encompassed all the analyzed probes on the chromosome suggesting a total loss of the chromosome. The chromosome 22 aberrated group was comparable to the entire cohort with regards to sex distribution, GKRS exposure, age, tumor volume and time elapsed from GKRS to surgery. Combining the structural variants identified in this study with whole-exome sequencing and multiplex ligation-dependent amplification (MLPA) data from our previous study, 41 out of 55 (75%) harbored at least one *NF2* mutation [7]. When only including the samples that were analyzed with whole-exome sequencing, 38 out of 46 (83%) harbored at least one mutation including 13 samples with one hit and 25 samples with 2 hits.

Mosaic chromosome 22 loss reveals intratumor genetic heterogeneity in sVS

Among the chromosome 22 aberrated tumors, a median aberrant cell fraction of 0.59 (0.25–0.94) was observed (Table 2). Aberrant cell fraction did not correlate with tumor growth. The level of mosaicism is demonstrated in the splitting of the BAF signal (Fig. 2). A Chi-square test demonstrated that tumors with aberrated chromosome 22 were significantly more likely to be estimated as heterogeneous ($\chi^2 = 22.212$, *Fisher's* $p = 0.000$). Among the four tumors with diploid chromosome 22 and estimated aberrant cell fraction below 1, one tumor had several CNN-ROHs, one tumor harbored another large CNA [del [21] (q11.2q22.3)], whereas the two other tumors were estimated to have an aberrant cell fraction between 0.95 and 1. It seems likely that ASCAT and Sequenza are dependent on a large CNA to estimate aberrant cell fraction and hence only estimates from chromosome 22 aberrant tumors were included for reporting (Table 2). In four chromosome 22 aberrated tumors, an aberrant cell fraction could not be estimated, and all these tumors had WES data only.

Among the 46 tumors analyzed with WES, a total of 45 small nucleotide variants and indels were found with a median variant allele frequency of 24% [7]. We found a positive correlation between the estimated aberrant cell fraction from the chromosome 22 aberrated tumors and variant allele frequency (adj $R^2 = 0.43$, $p = 0.006$).

GKRS does not alter the copy number profile of sVS

A Chi-square test of independence demonstrated no difference in the frequency of chromosome 22 aberration in irradiated (39%) and radiation-naïve (49%) tumors ($\chi^2 = 0.155$, *Fisher's* $p = 0.572$). We found no differences in aberrant cell fraction, number of CNAs, type of CNA or the portion of the genome covered by CNAs between the irradiated and radiation-naïve tumors. The clustering algorithms applied demonstrated that clusters identified did not rely on previous radiation exposure (Fig. 3). Using

Table 1 Patient demographics. Patient demographics of 55 vestibular schwannomas

ID	GKRS[1]	Age	Volume[2]	Sex	Microarray[3]	WES[4]
VS1	na	58	4.25	F	X	X
VS2	na	61	NA	M	X	X
VS3	na	68	16.34	M	X	X
VS4	na	67	3.17	F	X	X
VS5	na	58	12.82	M	X	X
VS6	na	57	NA	F	X	X
VS7	na	62	6.46	F	X	X
VS8	na	54	3.34	F	X	X
VS9	na	75	17.71	F	X	X
VS10	699	50	NA	F	X	X
VS11	1028	61	NA	M	X	X
VS12	3478	58	NA	M	X	X
VS13	1084	66	NA	M	X	X
VS14	2170	28	1.20	F	X	X
VS15	1079	64	NA	M	X	X
VS16	574	66	11.46	M	X	X
VS17	560	53	1.46	F	X	X
VS18	2371	69	1.67	F	X	X
VS19	1499	61	0.12	M	X	
VS20	280	44	0.62	M	X	
VS21	1476	66	3.50	M	X	
VS22	2968	80	0.41	M	X	
VS23	1987	60	0.47	M	X	
VS24	720	72	1.03	F	X	
VS25	811	68	0.14	M	X	
VS26	1646	61	4.56	F	X	
VS27	1288	61	2.52	M	X	
VS29	na	64	11.39	F		X
VS30	na	39	11.99	F		X
VS31	na	40	8.77	F		X
VS33	na	59	7.11	M		X
VS34	na	33	6.89	M		X
VS35	na	30	15.05	M		X
VS36	na	45	5.41	F		X
VS37	na	48	4.29	M		X
VS38	na	18	16.61	F		X
VS39	na	58	9.70	F		X
VS40	na	42	9.39	M		X
VS41	na	25	12.18	F		X
VS42	na	45	18.42	F		X
VS43	na	36	18.01	F		X
VS44	na	58	7.87	F		X
VS45	na	60	26.77	F		X
VS46	na	33	11.68	M		X
VS48	na	42	5.46	M		X
VS49	na	54	18.21	M		X
VS50	na	63	3.20	M		X
VS51	na	47	NA	M		X
VS52	na	55	6.80	M		X
VS53	na	66	8.57	M		X
VS54	na	37	12.94	F		X

Table 1 (continued)

ID	GKRS[1]	Age	Volume[2]	Sex	Microarray[3]	WES[4]
VS55	na	26	10.40	F		X
VS56	na	57	10.66	M		X
VS57	na	63	6.73	M		X
VS58	na	42	5.01	F		X

[1]Time in days between Gamma Knife Radiosurgery and microsurgery

[2]Tumor volume in cm^3

[3]Samples with DNA microarray data marked with X

[4]Samples with WES data marked with X

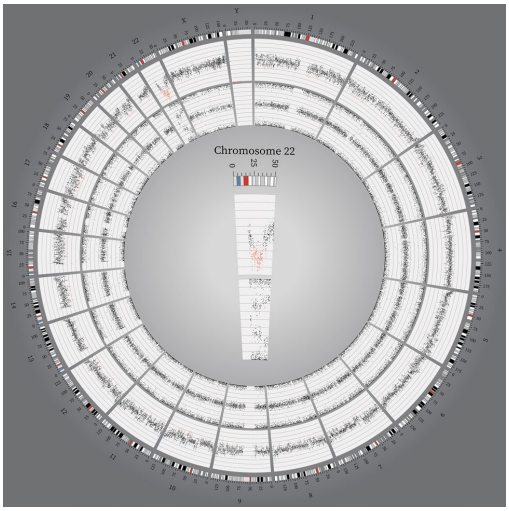

Fig. 1 Karyogram for sample VS10. Circos plot of copy number and single nucleotide polymorphism probe data for sample VS10, created using the Circos software [37]. The tracks from outside inwards: chromosome numbers, chromosomal position in Mb, copy number and allele patterns. Copy number gains and losses are highlighted in blue and red, respectively. Most chromosomes show a continuous disomic copy number profile with a normal three band allele pattern (allele configurations AA, AB and BB). On chromosome 22, highlighted in the middle, we see an allelic loss (allele configuration A0 and B0) in the region of *NF2* followed by a CNN-ROH (allele configuration AA and BB). However, the aberrations are only present in 63% of the cells giving rise to the split in the middle line of the allele pattern

the Nexus Copy Number built-in comparison analysis, we did not identify any CNA or gene associated with radiation exposure. We sought specifically for CNAs affecting genes coding for enzymes annotated to function in DNA repair pathways. Using the union call set, four tumors (two irradiated and two radiation-naïve) harbored CNAs affecting DNA repair genes. No difference between the groups was seen ($x^2 = 0.04$, $p = 0.58$). The results were similar for the ASCAT call set.

Table 2 Chromosome 22 aberrations

ID	Aberration	Aberrant cell fraction
VS1	22q11.1q13.33(16052530–51244019) × 1	0.86
VS8	22q11.1q13.33(16052530–51244019) × 1	0.46
VS9	22q11.1q13.33(17922735–51244019) hmz	0.46
VS10	22q11.1q13.33(19639383–37988033) × 1 22q11.1q13.1(37988034–51244019) hmz	0.63
VS13	22q11.1q13.33(16052530–51244019) × 1	0.61
VS16	22q11.1q13.33(16052530–51244019) × 1	0.46
VS17	22q11.1q13.33(16052530–51244019) × 1	0.29
VS20	22q11.1q13.33(16052530–51244019) × 1	0.26
VS22	22q11.1q13.33(16052530–51244019) × 1	0.45
VS27	22q11.21q13.33(18581773–51244019) hmz	0.25
VS33	22q11.1q13.33(16157603–51220938) × 1	0.63
VS34	22q11.1q13.33(16157940–51237063) × 1	0.59
VS37	22q11.1q13.33(16157827–51220938) hmz	0.36
VS38	22q11.23q12.3(24167473–33156768) hmz	NA
VS42	22q11.1q13.33(16157762–51220938) × 1	0.27
VS43	22q11.1q13.33(20761063–51220938) × 1	0.94
VS45	22q11.1q13.33(16157623–51237063) × 1	0.79
VS46	22q11.22q13.33(22313733–51237063) hmz	NA
VS50	22q11.1q13.33(16157622–51237063) × 1	0.77
VS51	22q11.1q13.33(16157603–51219006) × 1	0.66
VS53	22q11.1q13.33(26688838–51237063) × 1	0.8
VS54	22q11.1q13.33(16157603–51237063) × 1	NA
VS55	22q11.1q13.33(16157771–51220938) hmz	NA
VS57	22q11.23q13.33(23523234–51220938) hmz	0.4
VS58	22q11.1q13.33(16269779–51216564) × 1	0.84

Chromosome 22 aberrations identified in VS. The naming of the aberrations starts with chromosome number followed by band, location in bp and type of aberration (x1 for hemizygous loss and hmz for copy number neutral run of homozygosity). The last column gives the fraction of cells harboring the aberration

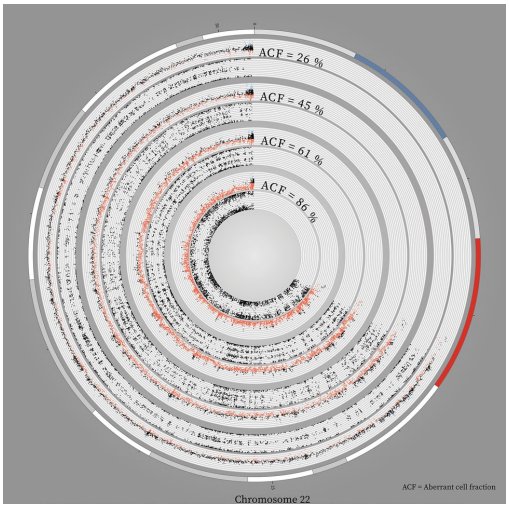

Fig. 2 Vestibular schwannoma consist of more than one major genetic clone. Circos plot of copy number and single nucleotide polymorphism probes in chromosome 22 for four vestibular schwannomas with increasing aberrant cell fraction. The tracks from outside inwards: chromosomal position in Mb on chromosome 22, copy number and allele patterns respectively for four vestibular schwannomas with increasing aberrant cell fraction. All samples demonstrate hemizygous loss of chromosome 22. The outermost sample shows a minor drop in copy number and a barely visible split in the middle line in the allele pattern because only 26% of the cells are aberrated. Moving inwards, the copy number drops and the split in the allele pattern increases, demonstrating an increase in aberrant cell fraction

Discussion

GKRS has become increasingly popular in treating VS over the past decades [21]. Several case reports have questioned its safety regarding malignant degeneration of benign tumors and inducing new neoplasms. For a review, see [9]. A dominating theory explaining the relationship between ionizing radiation dose and harmful effects is called the "linear no-threshold model" [22]. Although disputed, this theory explains that there is no safe limit, and that even a small amount of radiation might damage the DNA and initiate tumorigenesis. Standard treatment protocol with GKRS delivers 12 Gy to the periphery with a sharp decrease in the amount of energy delivered to the surrounding tissue. In the focus of the radiation, the dosage will be enough to initiate necrosis. In the periphery, we hypothesize that the dosage will harm the DNA and lead to one of the following: (1) detrimental DNA damage leading to apoptosis, (2) DNA mutations with tumorigenic potential, (3) DNA mutations with other or no effect at all. Although it seems theoretically feasible for GKRS to increase the risk of malignancy, epidemiologic studies do not support this [10, 23].

Previous studies have reported different genetic findings associated with radiation in VS. Lee et al. analyzed 30 sVS, including 4 irradiated tumors, utilizing microsatellite analysis to find that chromosome 22 aberration was more common in the radiation-naïve tumors [24]. Warren et al. found, using comparative genomic hybridization, that among 10 neurofibromatosis type 2 patients, radiation was associated with chromosomal aberrations [25]. In a recent study, Aaron et al. used WES on 12 VS, including two irradiated, to conclude that irradiated VS have increased copy number events and mutational burden [26]. One irradiated tumor harbored 184 mutations whereas the average across the cohort was 18.5. However, in our previous study utilizing WES on 46 VS, including 8 irradiated tumors, we also demonstrated one outlier, but this was radiation-naïve [7]. Taken together, this weakens the association between radiation exposure and hypermutated tumors.

This is the largest study investigating the effect of GKRS on the sVS genome. Using hierarchical clustering of the

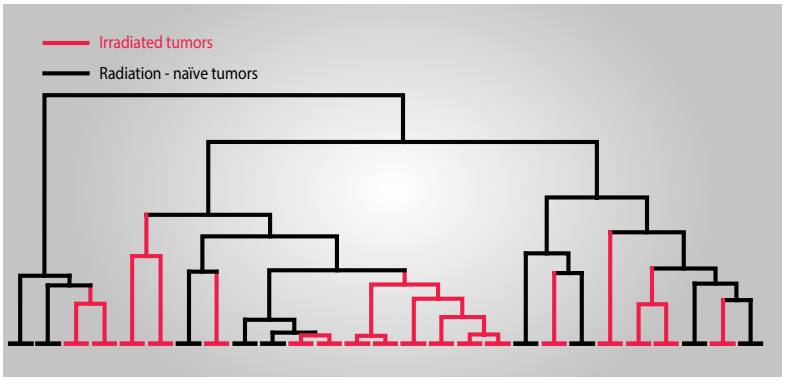

Fig. 3 GKRS treatment does not affect the genomic CNA profile of vestibular schwannoma. Dendrogram of hierarchical clustering of the autosomal CNA patterns of vestibular schwannomas. Irradiated and radiation-naïve tumors depicted as red and black terminal vertical lines respectively. The clusters are not associated with previous radiation exposure

genome-wide CNA profiles, we did not identify any clustering based on radiation exposure. Neither did we find any gene or genomic loci that correlated with radiation exposure. The discrepancy with previous studies on the subject might be due to larger sample size and the method used. In a recent paper, 18 radiation-induced meningiomas were analyzed for tumor-specific CNAs [27]. A mean total of 22% of the exome was affected by CNA. This is in stark contrast with our irradiated tumors exhibiting a median of 0.14% of the autosome covered by CNA. The meningioma patients had received cranial radiotherapy for diseases like medulloblastoma and central nervous system lymphoma, a therapy that delivers higher radiation doses to healthy tissue compared to GKRS. This comparison lends support to the fact that GKRS does not induce collateral damage to the extent seen after conventional radiotherapy. We expect that GKRS causes mutations in the normal tissue surrounding VS. However, the mutations induced need to provide a selective growth advantage to the affected cell initiating a clonal expansion for it to be detected using bulk DNA analysis and even for it to be clinically relevant. It is feasible that a growth advantage might be obtained, but that it is very rare in agreement with epidemiologic studies and our study.

Between 5 and 10% of sVSs do not respond to GKRS treatment. The GKRS response might depend on both treatment and tumor factors. Studying the tumor factors might elucidate the mechanism of radioresistance as well as identify biomarkers. Archibald et al. found a higher expression of the immune-related protein B7-H1 among irradiated sVS, but no difference at RNA level [28]. This might be a consequence of the radiation induced inflammation and hence not connected to the cause of the radioresistance. Through the use of genome-wide association studies, gene expression and DNA sequencing, several biomarkers of radiotherapy treatment response have been found in neoplasms [29–32]. It has been postulated that enhanced DNA repair mechanisms lead to radiotherapy treatment failure. Hence, we sought to evaluate whether the radioresistant VSs harbored CNAs in DNA repair genes. Although we identified some impaired DNA repair genes, they were distributed equally among the irradiated and radiation-naïve tumors. We did not find any other gene or genomic loci associated with radioresistance. One pitfall of our study is that we do not have positive controls for tumors that respond to GKRS treatment as these are not surgically removed. However, as GKRS treatment is effective in 90–95% of sVSs, we believe that the radiation-naïve tumors included in this study serve as a viable surrogate for GKRS treatment responders. Also, to detect any genetic aberrations caused by ionizing radiation, a longitudinal study design using paired samples of radiation-naïve and irradiated tumors would be the most sensitive. However, we believe our study design would be able to detect any large effects ionizing radiation.

Among the chromosome 22 aberrated tumors, a median of 59% of the cells harbored the CNA, suggesting that VS consists of more than one major clone. This is in accordance with our previous study on small mutations, where we reported a median *NF2* variant allele frequency of 24% [7]. Considering the bias of ASCAT only calling heterogeneity in tumors with large aberration and the variant allele frequencies reported in our WES study, it seems likely that most or all VSs consists of more than one major genetic clone. A recent study by Lewis et al. found that tumor-associated macrophages constituted 50–60% of the cells in eight growing VSs [33]. Hence, infiltrating macrophages might constitute the clone coexisting with the neoplastic cells. Further on, Lewis et al. found that macrophages accounted for the proliferating cells in VS. However, we did not find any association between aberrant cell fraction, which might correlate inversely with the macrophage fraction, and tumor growth. The finding of intratumor genetic heterogeneity has significant implications for molecular studies on VS. We saw from our data that the lower the aberrant cell fraction, the higher the number of CNAs called. This implies a problem with the software and theoretic framework underlying calling of aberrations. The possibly large fraction of infiltrating macrophages would also preclude other molecular studies using bulk analyzing, like transcriptome and proteome studies. A possible way to bypass this could be to apply single-cell analysis to provide a better understanding VS molecular biology.

Carlson et al. recently profiled structural variants in sVS using whole-genome sequencing to find biallelic inactivation of the *NF2* gene in all 22 sVSs analyzed [8]. Previous studies have demonstrated *NF2* variants in 15–84% of the analyzed tumors [34]. Combining our data from whole-genome microarray, WES and MLPA, we found at least one variant in *NF2* in 83% of the tumors. This discrepancy might reflect differences in the detection limit of the methods used or variations in the study population. Carlson et al. also found that VSs with severe phenotype tended to harbor large structural variants outside chromosome 22. However, we did not find any association with specific CNAs and previous radiation exposure, tumor size or age. In agreement with Carlson et al. we did not find any recurrent focal alterations and it seems unlikely that this kind of genetic event plays a significant role in VS tumorigenesis [8]. Previous studies have identified recurrent non-chromosome 22 regions affected by CNA, like 9q34, 17q, 19, 16q and 9p21 [25, 35]. We found a total of 38 genomic loci affected in three or more tumors. However, all but chromosome 22 aberration were normal variants present in healthy subjects.

In our previous study, we analyzed a total of 46 VS, including 8 irradiated VS, with WES and MLPA to conclude that radiation exposure or radiosensitivity is not associated with increased mutational burden or specific small

mutations [7]. Ionizing radiation is known to induce DNA double-strand breakage resulting in CNA [36]. To address this issue, we analyzed an extended set of irradiated samples with whole-genome DNA microarray to conclude that neither specific CNAs, nor the genomic CNA profile play a role either. The methods used in these studies are not capable of detecting structural variants not affecting gene dosage or heterozygosity (e.g. inversions and translocations). Hence, future studies should address this as well as epigenetic mechanisms to elucidate the molecular consequences of ionizing radiation in VS as well as markers of radioresistance.

Conclusions

We did not find any CNA or genomic CNA profile associated with radiation exposure in VS. This finding lends support to the low risk of GKRS. We demonstrated that VS exhibit intratumor heterogeneity and further studies are warranted to elucidate whether it is different tumor clones or normal cell infiltration. In our study, the only recurrent CNA in VS is hemizygous loss or copy number neutral loss of heterozygosity on chromosome 22.

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