AWARD NUMBER: W81XWH-19-1-0334

TITLE: Detecting Missing Heritability for Risk Stratification and Clinical Management of the Neurofibromatoses

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REPORT DATE: July 2022

TYPE OF REPORT: Annual

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
July 2022	Annual	01Jul2021-30Jun2022

4. TITLE AND SUBTITLE

Detecting Missing Heritability for Risk Stratification and Clinical Management of the Neurofibromatoses

5a. CONTRACT NUMBER
W81XWH-19-1-0334

5b. GRANT NUMBER
NF180020

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Neurofibromatosis type 1 (NF1), NF2 and schwannomatosis are genetically distinct tumour predisposing conditions, with significant clinical overlap, but differing prognoses and clinical management requirements. NF2 was recently re-grouped with the schwannomatoses due to its clinical phenotype. We are using a range of genetic techniques to identify the missing heritable elements in our cohort with NF1 or NF2/schwannomatosis and to stratify their genetic risk.

In our third year, we have published our genetic screen of schwannomatosis patients for potential causative genes. This study clarified that the candidate genes, *COQ6*, *DGCR8* and *CDKN2A/B*, which have recently been associated with schwannomatosis, are very rare contributors to this disorder. We have also carried out a genotype-phenotype study of *NF2* whole gene deletions and large intragenic deletions, duplications and rearrangements. We are currently working on some supporting functional work for this project prior to publication. In parallel, we have carried out long-read sequencing on a patient with a complex NF2 rearrangement to clarify the precise breakpoints and the overall level of complexity. We have also carried out deep sequencing on an initial cohort to assess the utility of our new gene panel design. Functional studies on untranslated *SMARCB1* variants are also ongoing.

15. SUBJECT TERMS

Neurofibromatosis, Schwannomatosis, NF1, NF2, SMARCB1, LZTR1, pathogenic variant, genetic predisposition, genetic diagnosis

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
Unclassified	Unclassified	Unclassified	Unclassified	29	19b. TELEPHONE NUMBER *(include area code)*

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1. **INTRODUCTION:**

Neurofibromatosis type 1 (NF1), NF2 and schwannomatosis are autosomal dominant neurogenetic disorders that predispose affected individuals to develop tumours of the nervous system. While these disorders are both clinically and genetically distinct, there are many cases with significant clinical overlap, particularly between NF2 and schwannomatosis. In fact, NF2 has recently been re-grouped as a member of the schwannomatoses (designated *NF2*-related schwannomatosis), as neurofibromas are not a part of the clnical phenotype. The new diagnostic criteria guidelines also highlight the importance of genetic diagnosis, as these disorders have different prognoses and have different clinical management requirements. Routine genetic testing currently identifies >90% of non-mosaic pathogenic variants in *NF1* and *NF2* genes, but there remain families with a clinical diagnosis of NF1 or *NF2*-related schwannomatosis for which no causative variant has been identified. In addition, the two additional major schwannomatosis predisposition genes, *SMARCB1* and *LZTR1*, currently only account for around 50% of non-*NF2*-related schwannomatosis cases. We have been using an extended range of genetic techniques to identify the underlying cause of disease in well characterized families with a clinical diagnosis of neurofibromatosis of schwannomatosis, in whom no underlying pathogenic mutation has yet been found. We have also investigated the mechanism of action of these variants and how specific variants can be stratified by disease risk.

2. KEYWORDS: Neurofibromatosis, Schwannomatosis, *NF1, NF2, SMARCB1, LZTR1*, pathogenic variant, genetic predisposition, genetic diagnosis

3. ACCOMPLISHMENTS:

What were the major goals of the project?

The major goals for year 3, were to:

1. Screen mutation-negative sporadic samples to assess frequency of newly identified pathogenic variants for each disease (30% complete).
2. Functional assessment of novel variants, depending on the type of variants identified in earlier analyses (50% complete).
3. Wrap up studies, final analysis of results and writing-up for publication (40% complete).

Pandemic re-organised tasks:

1. Publish results of schwannomatosis candidate gene screen.(100% complete)
2. Genotype-phenotype assessment of large *NF2* copy number variants for risk stratification (75% complete)
3. PacBio long-range sequencing on samples with complex NF2 rearrangements.(80% complete. Postponed from year 2)
4. Targeted next generation sequencing.(50% complete. Postponed from year 1 and re-designed due to the pandemic)
5. Functional investigation of *SMARCB1* 3'untranslated region. (75% complete, but investigations extended due to publication of a similar study (detailed below))

What was accomplished under these goals?

In year 3 we published our study which screened novel schwannomatosis candidate genes in a panel of patients with no identified pathogenic variant (PV) in *NF2*, *SMARCB1* or *LZTR1* (See author's accepted manuscript in Appendix 1). In summary, we carried out sequencing of the genes: *COQ6*, *DGCR8* and *CDKN2A/B*, which have all been recently associated with schwannomatosis in rare cases, with the aim of determining the frequency of PVs in these genes in a cohort of 75 schwannomatosis families. We found no PVs in *COQ6* or *DGCR8* in our cohort and only one *CDKN2A/B* variant in one patient. However, *CDKN2A/B* variants have also been associated with melanoma and the patient with the *CDKN2A/B* variant also had a history of melanoma. In addition, it is known that schwannomatosis patients previously identified with a germline *DGCR8* variant also had additional diagnoses, indicating complex diagnoses. Therefore, we hypothesise that variants in these three novel candidate genes are not common in, or specific to, schwannomatosis disease, but may cause schwannomas as part of more complex disease phenotypes.

This year, to stratify the risk of a sub-group of *NF2* variants, we have also carried out a genotype-phenotype study of *NF2* whole gene deletions, large intragenic deletions, duplications and rearrangements. The majority of *NF2* PVs are single nucleotide variants including nonsense, frameshift, splice-site and missense variants and previous genotype-phenotype correlations for single nucleotide variants have shown that the location of these variants correlates with disease severity. In general, variants that occur towards the 5' end of the gene cause a more severe clinical phenotype than those that occur towards the 3' end. Approximately 20% of *NF2* PVs are whole gene deletions, or large intragenic duplications, deletions, or rearrangements. These variants generally lead to a milder disease phenotype than truncating single nucleotide (nonsense or frameshift) variants. According to current clinically applied genetic severity scoring system, all large deletions that include the promoter or exon 1 are classified as mild, while large deletions that do not include the promoter or exon 1 are classified as moderate. The consequences of inversions and rare complex rearrangements is less clear. To investigate this genetic sub-class of variants further, we assessed genotype-phenotype correlations in our large cohort of 216 affected individuals with a known germline *NF2* pathogenic variant involving a whole gene deletion, large intragenic deletion, duplication or inversion, or a large rearrangement.

We found that disease severity correlated with starting exon of the large variants, similar to the pattern seen for single nucleotide variants (Figure 1). Whole gene deletions led to the mildest phenotype. Large intragenic deletions starting in exon 1 resulted in a more severe phenotype than whole gene deletions, but milder than variants starting in exons 2 to 9, which led to the most severe phenotypes. Variants that started in exons 10-15 were comparable in severity to whole gene deletions. Our results suggest the possibility that not all large deletions lead to a complete loss of expression, despite disrupting larger proportions of the gene.

Figure 1. Cartoon indicating *NF2* exon boundaries superimposed over Merlin protein functional domains. Light pink exons indicate the mildest form of disease is generally associated with deletions starting in these exons. Darker pink indicates a moderate disease phenotype and red indicates the most severe phenotypes.

In order to investigate this hypothesis further, we carried out RNA analysis on patient-derived lymphoblast cell lines known to contain specific deletions or rearrangements to identify aberrant RNA transcripts (Figure 2). We identified three aberrant transcripts, two of which were out-of-frame and are predicted to undergo nonsense-mediated decay. One sample identified an in-frame transcript with exons 2-10 deleted.

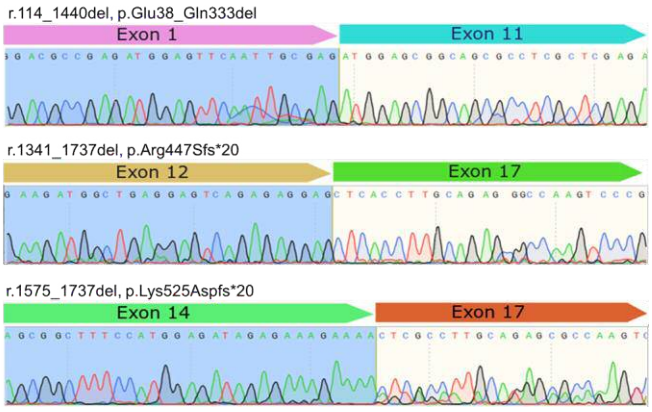

Figure 2. Chromatograms showing results of RNA analysis of three patients with large *NF2* alterations.

The stability of these aberrant transcripts is unknown. However, Western blotting analysis did not identify expressed protein for any of the aberrant transcripts, despite the antibody being know to bind in a region that is predicted to be present in the in-frame aberrant transcript. We are currently working on some supporting functional work for this project prior to publication. We are in the process of generating expression constructs containing these aberrant transcripts to determine their relative stability (using qPCR). This will help to determine whether the stability of aberrant RNA transcripts correlates with disease severity in these patients.

This year we have also carried out PacBio long-read sequencing of a sample with a complex *NF2* rearrangement to clarify the precise chromosomal breakpoints involved in the rearrangement and the overall level of complexity. The sequencing data is

currently being analysed and will be incorporated into a publication to determine the detection rate of *NF2* pathogenic variants using a combination of multiple genetic screening techniques.
Initial SNP array analysis of familial samples did not identify any clear regions of copy number change in their germline DNA. Therefore, we have also carried out high read-depth next generation sequencing on an initial cohort of NF1, NF2 and schwannomatosis patients to assess the utility of our new gene panel design to identify novel/non-coding variants and/or previously missed mosaic variants in the known causative genes. Due to the pandemic, prices for genetic sequencing reagents and services have significantly increased and our original strategy became unaffordable. Therefore, we have subsequently re-scoped this part of the project to accommodate these changes. We have now re-designed our gene panel and submitted the first set of samples for sequencing. This sequencing data is currently being analysed.

Functional studies on untranslated *SMARCB1* variants are also ongoing. We have continued with experiments previously reassigned to year 1, including functional assessment of *SMARCB1* 3'UTR variants of uncertain significance. We completed molecular cloning of *SMARCB1* expression constructs and subsequent luciferase assays (Figure 3) to compare expression levels of mutant *SMARCB1* 3'UTRs with the wild type transcript.

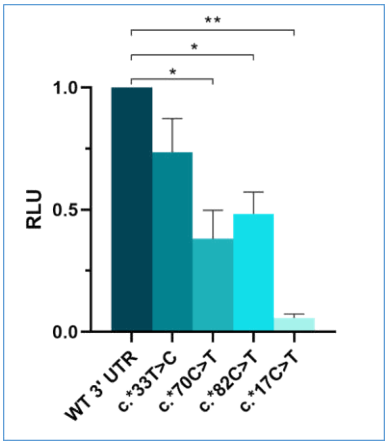

Figure 3. Results of luciferase expression analysis of *SMARCB1* 3'UTR constructs transfected into a wild type schwann cell line. The mutant 3'UTR constructs cause reduced expression of the *SMARCB1* transcript, by different amounts. RLU=Relative Light Units, WT 3'UTR=wild type 3'-untranslated region.

Having completed these assays, a similar study was published by Piotrowski et al, (Human Mutation, 2021). Our results are similar, but not identical to the results in the published study. Therefore we decided that further investigation would be necessary to identify the cause of the differences between our results and those of Piotrowski et al. We aim to determine if the differences are due to experimental/methodological differences, and which results are most representative of expression levels seen in schwannomatosis patients with these variants.

Since demonstrating that the *NF2* 5'UTR variant c.-66_-65insT affects NF2 protein translation via interfering with an upstream open reading frame (uORF), published by Whiffin et al 2020, we have changed our strategy for investigating this variant. We have carried out western blotting to detect any reduced expression of NF2 protein in a lymphocyte protein extract. However, no reduced expression was observed. We therefore decided that in order to investigate this variant further a new study will be necessary and Dr Perrez-Becerril is currently applying for a New Investigator Research Award incorporating a strategy to create this variant in heterozygous and homozygous form in an immortalised schwann cell line using a CRISPR genome editing approach. This model can then be used to assess the variant in greater detail.

- **What opportunities for training and professional development has the project provided?**

The PDRA, Dr Perez-Becerril, has continued to undertake relevant training courses provided by the University. She has attended regular lab meetings, departmental seminars and has given presentations at journal clubs and at the Children's Tumor Foundation annual NF conferences. She has had experience of writing a review article and a research article and is currently applying for a New Investigator Award to continue her work after the current funding period ends.

- **How were the results disseminated to communities of interest?**

So far, we have published a review article and two research articles (in the journals Genetics in Medicine and Human Mutation). Dr Perez-Becerril and Dr Smith have also presented new data each year at University research meetings and at the Children's Tumor Foundation annual NF meeting, which is attended by scientists, clinicians, patient representatives and other interested parties with an interest in the neurofibromatoses.

- **What do you plan to do during the next reporting period to accomplish the goals?**

In the next reporting period we plan to: 1) complete the remaining functional work for the genotype-phenotype risk stratification study of *NF2* large deletions, 2) complete the analysis of long read sequencing data for the complex *NF2* rearrangement, 3) complete gene panel testing for the remaining samples with unfound variants and 4) plan the additional functional analyses that will be required for assessment of *SMARCB1* 3'-UTR variants. We are also applying for further funding to expand on ideas that have arisen during the current funding period.

4. **IMPACT:**
   - **What was the impact on the development of the principal discipline(s) of the project?**

Nothing to report.

   - **What was the impact on other disciplines?**

Nothing to report.

   - **What was the impact on technology transfer?**

Nothing to report.

   - **What was the impact on society beyond science and technology?**

Nothing to report.

5. **CHANGES/PROBLEMS:**
   - **Changes in approach and reasons for change**

Due to the pandemic, prices for genetic sequencing reagents and services increased significantly and we re-scoped our high read depth sequencing project to accommodate these changes. We have now re-designed our gene panel and submitted the first batch of samples for sequencing. This data is now being analysed. We are also planning to expand our studies of *SMARCB1* 3'UTR variants due to the publication of a similar study which obtained similar, but not identical results (detailed above).

   - **Actual or anticipated problems or delays and actions or plans to resolve them**

The delays encountered during this reporting period have been due to the ongoing global pandemic, which has caused delays to the receipt of some laboratory reagents and other consumables. However, during this year laboratory based research has now resumed full time with normal levels of occupancy.

   - **Changes that had a significant impact on expenditures**

The ongoing global pandemic has affected year 3 spending. We have continued to reorganise the major research activities described in the SOW to accommodate the major changes made in years 1 and 2. We have also obtained a no cost extension to the award to allow us to complete the remaining work.

   - **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

No changes were made to the anticipated use of human samples. We obtained institutional Research Ethics Committee (REC reference 10/H1008/74; R&D Pin: R10257) prior to the start of the funding period.

   - **Significant changes in use or care of human subjects**

None.

   - **Significant changes in use or care of vertebrate animals.**

None.

   - **Significant changes in use of biohazards and/or select agents**

None.

6. **PRODUCTS:**
   - **Publications, conference papers, and presentations**
     - **Journal publications.**

Perez-Becerril C, Wallace AJ, Schlecht H, Bowers NL, Smith PT, Gokhale C, Eaton H, Charlton C, Robinson R, Charlton RS, **Evans DG, Smith MJ**. Screening of potential novel candidate genes in schwannomatosis patients. Hum Mutat. 2022 Jun 20. doi: 10.1002/humu.24424. Online ahead of print.

     - **Books or other non-periodical, one-time publications.** Nothing to report.
     - **Other publications, conference papers, and presentations.**

Conference poster presentation: **Smith MJ, Cristina Perez-Becerril,** Meenakshi Minnis, Naomi L Bowers, Claire L Hartley, Philip T Smith, Andrew T King, Simon K Lloyd, Scott A Rutherford, Omar N Pathmanaban, Charlotte Hammerbeck-Ward, Simon R Freeman, and **D Gareth Evans**. Genotype-phenotype correlations involving germline pathogenic copy number variants in the NF2 gene. Children's Tumor Foundation virtual NF conference. Philadelphia. June 18th-21th 2022.

Conference poster presentation: **Perez-Becerril C**, Burghel G, Hartley CL, **Evans DG** and **Smith MJ**. Identification and characterisation of a large structural variant in a family affected by NF2-schwannomatosis. Children's Tumor Foundation virtual NF conference. Philadelphia. June 18th-21th 2022.

   - **Website(s) or other Internet site(s)**
     N/A
   - **Technologies or techniques**
     N/A
   - **Inventions, patent applications, and/or licenses**
     N/A
   - **Other Products**
     N/A

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**
   - **What individuals have worked on the project?**

Name:	Miriam J Smith
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	https://orcid.org/0000-0002-3184-0817
Nearest person month worked:	3

Contribution to Project:	Dr. Smith has trained the PDRA, coordinated the project work, submitted ethics applications, and been involved in writing up of journal articles. She has also given platform presentations at the Children's Tumor Foundation annual NF conference.
Funding Support:	N/A

-

Name:	Cristina Perez-Becerril
Project Role:	PDRA
Researcher Identifier (e.g. ORCID ID):	https://orcid.org/0000-0003-1630-1943
Nearest person month worked:	12
Contribution to Project:	Dr. Perez-Becerril has undertaken relevant training courses, attended relevant seminars. She has also presented posters at the Children's Tumor Foundation annual NF conferences. She has written a review article and a research article both published in Human Mutation. She has screened a cohort of schwannomatosis patients for a panel of candidate causative genes, and she is continuing her molecular cloning work/functional assays and large-scale sequencing for the project.
Funding Support:	N/A

Name:	Prof D. Gareth Evans
Project Role:	Co-I
Researcher Identifier (e.g. ORCID ID):	https://orcid.org/0000-0002-8482-5784
Nearest person month worked:	1
Contribution to Project:	Professor Evans has advised on clinical aspects of the project work and provided clinical details on patient samples.
Funding Support:	N/A

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Nothing to report

- **What other organizations were involved as partners?**
  - **Organization Name:** National Health Service
  - **Location of Organization:** Manchester
  - **Partner's contribution to the projec**t: Our laboratory space is housed within a National Health Service building.
  - **Financial support;**
    - **Facilities** Project staff use the partner's facilities for project activities.

8. **SPECIAL REPORTING REQUIREMENTS**
   - **COLLABORATIVE AWARDS:** N/A
   - **QUAD CHARTS:** N/A
9. **APPENDICES:**

Appendix I: Authors' accepted version of publication:

Perez-Becerril C, Wallace AJ, Schlecht H, Bowers NL, Smith PT, Gokhale C, Eaton H, Charlton C, Robinson R, Charlton RS, **Evans DG, Smith MJ**. Screening of potential novel candidate genes in schwannomatosis patients. Hum Mutat. 2022 Jun 20. doi: 10.1002/humu.24424. Online ahead of print. PMID: 35723634

Screening of potential novel candidate genes in schwannomatosis patients

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Funding Information

USAMRAA CDMRP Neurofibromatosis Research Program, Investigator-Initiated Research Award (W81XWH1910334); Manchester National Institute for Health Research (NIHR) Biomedical Research Centre (IS-BRC-1215-20007).

Abstract

Schwannomatosis comprises a group of hereditary tumour predisposition syndromes characterised by, usually benign, multiple nerve sheath tumours, which frequently cause severe pain that does not typically respond to drug treatments. The most common schwannomatosis-associated gene is *NF2*, but *SMARCB1* and *LZTR1* are also associated. There are still many cases in which no pathogenic variants (PVs) have been identified, suggesting the existence of as yet unidentified genetic risk factors. In this study, we performed extended genetic screening of 75 unrelated schwannomatosis patients without identified germline PVs in *NF2*, *LZTR1*, or *SMARCB1*. Screening of the coding region of *DGCR8*, *COQ6*, *CDKN2A* and *CDKN2B* was carried out, based on previous reports that point to these genes as potential candidate genes for schwannomatosis. Deletions or duplications in *CDKN2A*, *CDKN2B* and adjacent chromosome 9 region were assessed by multiplex ligation-dependent probe amplification analysis (MLPA). Sequencing analysis of a patient with multiple schwannomas and melanomas identified a novel duplication in the coding region of *CDKN2A,* disrupting both *p14ARF* and *p16INK4a*. Our results suggest that none of these genes are major contributors to schwannomatosis risk but the possibility remains that they may have a role in more complex mechanisms for tumour predisposition.

Keywords

Schwannomatosis screening, candidate genes, *DGCR8*, *COQ6, CDKN2A*, *CDKN2B*

Introduction

Schwannomatosis comprises a group of autosomal dominant tumour predisposition syndromes characterised by the development of multiple schwannomas. The most common form is associated with the *NF2* gene, but at least two further genetically distinct forms exist. Causative variants for non-*NF2*-related schwannomatosis have been primarily identified in two genes; *SMARCB1* (SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily b, member 1) and *LZTR1* (leucine zipper like transcription regulator 1), both located in the chromosome 22q region although these variants only account for 30 – 40% of sporadic cases and 70 – 80% of familial cases (Evans et al., 2018; Hulsebos et al., 2007; Kehrer-Sawatzki et al., 2017; Piotrowski et al., 2014). In addition, the majority of non-*NF2*-related schwannomatosis cases are sporadic (MacCollin et al., 2005), suggesting the existence of novel schwannomatosis variants and/or genes.

Previous studies have proposed a role for additional genes in the pathogenesis of schwannomatosis. Whole exome sequencing analysis of 10 Korean sporadic schwannomatosis patients, identified 26 variants of which 13 were predicted to be pathogenic from *in silico* analysis. One of these potentially pathogenic variants (PVs) was a missense change (NM_000077.4:c.85C>A; p.Ala29Ser) located in exon 1 of the cyclin dependent kinase inhibitor 2A (*CDKN2A*) gene, in the chromosome 9p21.3 region (Min et al., 2020).

CDKN2A encodes two proteins, p16INK4a and the alternatively translated p14ARF, both of which have a role in tumour suppression, through regulation of Rb and p53 pathways (Quelle et al., 1995; Zhang et al., 1998a; Zhang et al., 1998b). Loss of function of both *CDKN2A* and its tandemly linked gene *CDKN2B*, which encodes p15INK4b, another regulator of the Rb

pathway (Hannon et al., 1994), have been implicated in a variety of cancers from central nervous system (CNS) tumours, including schwannomas (Ali et al., 2021; Almeida et al., 2008; Cancer Genome Atlas Research, 2008; Zhang et al., 1996) pancreatic cancer, renal cancer and melanoma (Goldstein et al., 2006; Jafri et al., 2015; McNeal et al., 2015; Patel et al., 2020; Tu et al., 2018). Indeed, *CDKN2A* is one of the main susceptibility genes for familial melanoma with both point mutations and gene deletions implicated in pathogenesis (Goldstein et al., 1997; Hussussian et al., 1994; Kamb et al., 1994; Pollock et al., 1998; Whiteman et al., 1997). In addition, a splicing variant in *CDKN2A* (NM_000077.4:c.151–1G>C), responsible for loss of p16INK4a and p14ARF has been reported in a number of families affected by multiple neoplasms, including nerve sheath tumours and melanomas (Petronzelli et al., 2001; Prowse et al., 2003; Sargen et al., 2016). Notably in the most recent of these reports, Sargen and colleagues observed that a number of nerve sheath tumours, across affected family members carrying the *CDKN2A* variant, presented features consistent with both schwannoma as well as neurofibroma histopathology.

Other proposed candidate genes for schwannomatosis include the coenzyme Q6, monooxygenase (*COQ6*) gene and DGCR8 microprocessor complex subunit (*DGCR8*). There has been one report of a constitutional missense variant in exon 6 of *COQ6* (NM_182476.2: c.622G>C; p.Asp208His) segregating with disease in a schwannomatosis affected family (Zhang et al., 2014). More recently, a study identified a germline variant in exon 7 of *DGCR8* (NM_022720.6:c.1552G>A; p.Glu518Lys) in all affected members of a family with both euthyroid multinodular goitre (MNG) and schwannomatosis (Rivera et al., 2020). This variant, which was predicted to be pathogenic by a number of algorithms and by *in silico* models, was subsequently characterised to determine its role in disruption of micro RNA (miRNA)

biogenesis. Furthermore, a recent analysis of 13 schwannomas from patients affected by schwannomatosis and multinodular goiter identified the p.Glu518Lys pathogenic variant in *DGCR8* as the only germline pathogenic variant in four of these tumours (Nogué et al., 2022). All 13 tumours were found to have loss of heterozygosity (LOH) in the chromosome 22q region containing *DGCR8*, *LZTR1*, *SMARCB1* and *NF2.* For 5/13 tumours, all from the same individual, no other germline pathogenic variants in other schwannomatosis genes were identified and no somatic *NF2* variants were identified in 4/5 tumours resected from this patient. The authors propose a new model for schwannoma formation in which the inactivating mutation in *DGCR8* constitutes the first hit, whereas loss of the second *DGCR8* allele, along with *LZTR1*, *SMARCB1* and *NF2* constitute hits 2, 3, 4 and 5. For some of these tumour, a 6th somatic hit was also seen in *NF2* in the remaining 22q allele.

The purpose of this study was to assess the contribution of variants in *COQ6*, *DGCR8*, *CDKN2A* and *CDKN2B* to pathogenesis in a group of patients whose clinical features are consistent with schwannomatosis diagnosis, but for whom routine genetic analysis failed to identify PVs in the known schwannomatosis genes; *NF2*, *SMARCB1* and *LZTR1*. These individuals were also negative for germline chromosome 22q11.2 deletions, in order to confirm schwannomatosis diagnosis.

Materials and Methods

DNA extracted from lymphocytes of 77 schwannomatosis patients from 75 schwannomatosis families, from the local register at the Manchester Centre for Genomic Medicine was used for analysis. Demographic data for our cohort is summarised in Table 1. All patients included in the study met current clinical diagnostic criteria for schwannomatosis (Evans et al., 2018). These patients had also previously undergone routine genetic screening from which no PVs

in *NF2*, *SMARCB1* or *LZTR1* were identified. Routine analysis for schwannomatosis consists of screening of the coding region of *NF2*, *SMARCB1* and *LZTR1*, including 15 base pairs of intronic region at each side of exon-intron boundaries as well as part of the untranslated regions (UTRs) where PVs are known to occur. 42 patient samples in our cohort were received in or after 2013 and have been screened using next generation sequencing (NGS) with a mean coverage of 1000x for *NF2,* optimised for detection of mosaicism to a level of 5%. NGS analysis was also carried out at a read depth of 350x for *SMARCB1* and *LZTR1* on 46/77 and 52/77 patients respectively. For patients for whom *NF2* screening was carried out by Sanger sequencing only (35/77 individuals), one or more tumour samples were analysed when available (10/35). Clinical genetic testing techniques used for screening of index cases are summarised in Supp. Table S1. Genetic testing of two anatomically distinct tumour samples ruled out a diagnosis of mosaic NF2 for 2 of these patients (S. A summary of molecular testing of these tumours is presented in Supp. Table S2. The remaining 33/35 whose samples were collected prior to 2013, and did not undergo NGS screening for *NF2* variants, were classified as schwannomatosis patients based on current clinical diagnostic criteria (Evans et al., 2018), but mosaic NF2 has not been excluded genetically. A summary of clinical details and results from clinical genetic testing for patients in our cohort is provided in Supp. Table S2.

The presence of copy number variants (CNVs) is also routinely assessed through multiplex ligation-dependent probe amplification (MLPA) analysis of *NF2* (probe-set P044; MRC Holland), *SMARCB1* (probe-set P258; MRC Holland) and *LZTR1* (probe-set P455; MRC Holland). Finally, loss of heterozygosity (LOH) is assessed in tumour samples, when available, with *NF2* intragenic and flanking polymorphic microsatellite markers. Ethical approval for the study was obtained from the North West – Greater Manchester Central Research Ethics

Committee (reference 10/H1008/74). Research based sample screening and analysis were carried out under ethics approval (reference 10/H1008/74) obtained from the North West 7–Greater Manchester Central Research Ethics Committee. Patient data from large clinical databases was anonymized for this study.

Primers were designed to target flanking sites at each side of exons for regions of *COQ6* (NM_182476.2), *DGCR8* (NM_022720.6), *CDKN2A* (NM_000077.4) and *CDKN2B* (NM_004936.3) and are listed in Supp. Table S3. In addition, two intronic regions of *SMARCB1* known to harbour PVs for schwannomatosis (Piotrowski et al., 2021; Smith et al., 2020) were also screened. Sanger sequencing of amplicons was then carried out using BigDye™ Terminator v3.1 Cycle Sequencing Kit (ThermoFisher Scientific) and an ABI 3100 automated sequencer (Applied Biosystems).

Multiple ligation-dependent probe amplification (MLPA) was performed for 70 of the 77 samples (for which DNA was available), using the SALSA MLPA kit, P419 probemix (MRC-Holland, Amsterdam, The Netherlands) probe set from MRC Holland. Briefly, 100 ng DNA was used for the hybridization, ligation, and amplification of exon probes for control and test samples according to the manufacturer's instructions and analysed on an ABI 3100 automated sequencer (Applied Biosystems, Warrington, UK).

In silico analysis was performed for all variants identified in our cohort. Potential pathogenicity of missense variants was assessed using REVEL v4.2 (Ioannidis et al., 2016) and BayesDel v4.2 (Feng, 2017). Nonsense and intronic variants were assessed using CADD v1.4 (Rentzsch et al., 2019). Maximum credible population allele frequency (AF) values (Whiffin et al., 2017) and, when applicable, constrain metrics from gnomAD v2.1.1 (Karczewski et al., 2020) and DECIPHER v11.9 (Firth et al., 2009) were also used to aid in classification of variants

according to the American College of Medical Genetics and Genomics (ACMG) guidelines (Richards et al., 2015; Tavtigian et al., 2018) and the Association for Clinical Genomic Science (ACGS) best practice guidelines for variant classification in rare disease (Ellard et al., 2020). Potential splicing effects of variants were assessed using SpliceAI (Jaganathan et al., 2019), as well as MaxEntScan (Yeo et al., 2004), GeneSplicer (Pertea et al., 2001), NNSPLICE (Reese et al., 1997), EX-SKIP (Raponi et al., 2011), and SpliceSiteFinder-Like (Shapiro et al., 1987) as implemented in Alamut® Visual software.

Results

No pathogenic or likely pathogenic variants were identified in *DGCR8*, *COQ6* or *CDKN2B*. Bidirectional sequencing revealed a heterozygous single nucleotide duplication in exon 2 of *CDKN2A* (NG_007485.1:g.28291dup) in DNA from a patient with five nerve sheath tumours (two were considered hybrid tumours with high schwann cell content but some neurofibroma features). As the patient had no clinical features of NF1 and no vestibular schwannomas she was considered to have presumed schwannomatosis. Molecular analysis was carried out in one of the two independent hybrid tumours, found no PVs in *NF2*, *SMARCB1* or *LZTR1* and no evidence for loss of heterozygosity for *NF2* markers. This patient also had family history of melanoma (affected paternal grandfather and uncle) and previously presented with two melanomas, which were a malignant melanoma Clark stage 3 and a superficial spreading melanoma in situ. The duplication identified in this patient disrupted both isoforms of *CDKN2A*, which code for p16INK4a (NM_000077.4:c.158dup; p.Met53fs) and p14ARF (NM_058195.3:c.201dup; p.Asp68Ter) respectively. This is similar to a previous report of a splicing variant in *CDKN2A* (NM_000077.4:c.151–1G>C) that resulted in inactivation of both gene isoforms (Sargen et al., 2016) and which was observed in DNA samples from three

members of a family affected by melanoma and multiple nerve sheath tumours, some of which showed overlapping schwannoma and neurofibroma features.

The variant we identified in *CDKN2A* has not been reported previously but it is located in a highly conserved region and has been classified as pathogenic based on ACMG and ACGS guidelines (Figure 1). To investigate this duplication in schwannomatosis, we screened the coding sequence of *CDKN2A* in one schwannoma sample from this same patient. The NM_000077.4:c.158dup/ NM_058195.3:c.201dup was also present in heterozygous form. No other variants were identified, except previously reported polymorphisms. Previous analysis of this tumour sample revealed no evidence of 22q involvement. The variant was inherited from her unaffected father. Her paternal grandfather and uncle both had a history of melanoma; however DNA was not available from these family members for molecular testing.

MLPA analysis was carried out in 70 people for whom high quality DNA was available. No copy number abnormalities were detected in *CDKN2A* or *CDKN2B* for any of the samples analysed. A reproducible decrease in signal of a single control probe for the transmembrane channel like 1 (TMC1) on chromosome 9q21.13, was seen in a patient with thyroid cancer and schwannomatosis. Sequencing of the probe region found no known polymorphisms, suggesting the possibility of hemizygosity. Mutations in the *TMC1* gene have been associated with congenital and progressive hearing loss (Kurima et al., 2002) but no links to thyroid disorders or predisposition to nervous system tumours have been established. However, the significance of this result remains unclear. Previous analysis of schwannoma DNA identified LOH on chromosome 22, but subsequent MLPA analysis for *CDKN2A* failed for this sample.

Discussion

The predominant model of inheritance for familial schwannomatosis is a dominant model, similar to NF1 and NF2. However, whilst routine genetic analysis is able to identify around 92-95% of variants responsible for familial cases for NF1 and NF2, the proportion of familial schwannomatosis cases that can be attributed to known variants is much lower (70-80%). This proportion is even lower for sporadic schwannomatosis cases (30 – 40%) (Evans et al., 2018; Kehrer-Sawatzki et al., 2017). The genetic architecture of schwannomatosis appears to be more complex than that of NF1 and NF2 with constitutional *SMARCB1* PVs contributing in much higher proportion to familial schwannomatosis than they do to sporadic cases, whereas germline *LZTR1* PVs seem to contribute similarly to familial and sporadic cases (Evans et al., 2018). This has prompted increasing efforts to identify novel PVs for schwannomatosis, some of which might turn out to be located in hitherto undiscovered schwannomatosis loci.

Many of these efforts have benefitted from advances in technologies for genomic analysis. Whole exome sequencing (WES) of DNA from both sporadic and familial schwannomatosis samples have implicated a number of genes and variants of interest (Min et al., 2020; Rivera et al., 2020; Zhang et al., 2014). However, the extent to which these variants can be considered causative for schwannomatosis has proved to be harder to determine. In some cases, such as the recently reported germline variant in *DGCR8* (NM_022720.7:c.1552G>A; p.Glu518Lys), functional characterisation has provided strong evidence of the pathogenic nature of the variant. Additionally, clinical features of individuals carrying this variant prompted the authors to conclude that the variant might define a novel syndrome, characterised by the co-occurrence of schwannomatosis and familial multinodular goitre. Of note, only one of the schwannomatosis patients in our cohort had a thyroid related co-

morbidity, which was not classified as familial and no *DGCR8* variant was identified in this patient.

In contrast to *DGCR8*, evidence supporting the involvement of variants in *CDKN2A* and *COQ6* in schwannomatosis pathogenesis is less conclusive. Previous variants reported as potentially associated with schwannomatosis for these genes (Min et al., 2020; Zhang et al., 2014) have not been fully characterised, so the mechanisms through which they might contribute to disease remain unclear.

In the case of *COQ6*, there is need for more exhaustive functional analysis in order to establish a plausible mechanism through which dysfunction of *COQ6* might lead to schwannomatosis, particularly in the absence of evidence for bi-allelic inactivation of this gene in tumour tissue from affected members of the family in which the variant was originally identified. Indeed, questions have been raised about the role of this variant as a causative variant for schwannomatosis (Trevisson et al., 2015), since there were other variants that were identified by the same study that originally reported the *COQ6* variant, but that were not deemed of interest by the authors. To date, no other studies have identified *COQ6* variants in schwannomatosis patients, including our present study.

A link between malignant melanoma and nervous system tumours has been established by a number of studies. Particularly, germline whole gene deletions of *CDKN2A* and *CDKN2B* are known to be associated with familial syndromes predisposing to malignant melanoma as well as other nervous system tumours, including meningioma, astrocytoma and schwannoma (Azizi et al., 1995; Bahuau et al., 1998; Bahuau et al., 1997; Chan et al., 2017; Kaufman et al., 1993). At the somatic level, inactivation of *CDKN2A* and *CDKN2B* has been identified as an important feature in a number of tumours, most notably melanomas and tumours in the

central nervous system (Boström et al., 2001; Casula et al., 2019; Ghasimi et al., 2016; Gonzalez-Zulueta et al., 1995; McNeal et al., 2015; Rousseau et al., 2003). Interestingly, loss of CDKN2 proteins in meningiomas has been established as an important consideration for tumour classification and, in some cases, a determinant of tumour progression (Goutagny et al., 2010; Suppiah et al., 2019). This raises the possibility for a more prominent role of variants in *CDKN2A* and *CDKN2B* genes as modulators of clinical phenotypes. The role of *CDKN2A* dysfunction in the pathology of schwannomatosis has not been established but some clues may emerge from the study of the mechanisms involved in transformation of neurofibromas into malignant peripheral nerve sheath tumours (MPNSTs) resulting from bi-allelic inactivation of *CDKN2A* (Chaney et al., 2020; Magallón-Lorenz et al., 2021; Nielsen et al., 1999). These mechanisms appear to be relevant not only to MPNSTs tumour progression but also to multiple malignant and benign tumour predisposition (Sargen et al., 2016). It is therefore possible that the in *CDKN2A* we report here might be contributing to a similar complex syndrome.

The absence of germline PVs in *DGCR8*, *COQ6* or *CDKN2B* within a group of clinically well characterised schwannomatosis patients suggests that none of these genes is likely to be a major contributor to schwannomatosis pathogenesis on its own, although the possibility remains that they may have a role in complex clinical phenotypes. There is also a possibility that at least a proportion of the schwannomatosis cases that remain genetically unexplained might be caused by a variant within *NF2*, *SMARCB1* or *LZTR1* that has been missed by routine diagnostic methods. This could help explain the fact that previous studies using whole genome sequencing of schwannomatosis patients have either been unable to identify novel candidate genes (Hutter et al., 2014) or have reported potentially pathogenic variants in a

number of loci, which have not been validated (Min et al., 2020; Zhang et al., 2014). Indeed a deep intronic PV, NM_003073.5:c.795+1498C>T, leading to the inclusion of a cryptic exon and a truncated product, has previously been reported in intron 6 of *SMARCB1* (Smith et al., 2020). In addition, a recent deep massive parallel sequencing study of 35 schwannomatosis cases (Piotrowski et al., 2021) reported two novel deep intronic variants in intron 4 of *SMARCB1* (NM_003073.5:c.500+883T>G and NM_003073.5:c.500+887G>A). Both of these variants were further characterised by means of RNA analysis and demonstrated to result in retention of part of intron 4 and a truncated transcript. We have screened these intronic regions in our cohort and found that none of our patients is a carrier for any of these three variants. The variants in intron 4 were covered by our clinical NGS panel, but deep-intronic regions are not typically scrutinised for diagnostic purposes. The intron 6 variant was not captured by the panel. Furthermore, limitations of some of the techniques used in the past for genetic molecular testing might mean that for some individuals, low level mosaic variants in *NF2* have been missed. This in turn may result in misdiagnosis of mosaic NF2 cases as schwannomatosis, particularly for cases where there is limited availability of tumour tissue (Evans et al., 2018; Kehrer-Sawatzki et al., 2018). The current use of high read depth NGS analysis in clinical genetic testing has improved the rate of detection for mosaic pathogenic variants in *NF2* (Contini et al., 2015; Evans et al., 2020; Louvrier et al., 2018), however re-analysis of patient samples is not always possible. Future efforts to find novel PVs for schwannomatosis might be greatly aided by a similar approach to the one used by Piotrowski and colleagues, which involved deep sequencing of the full gene region of *NF2*, *SMARCB1* and *LZTR1*, which will help detect non-coding PVs that are difficult to identify by standard NGS panels or whole exome sequencing (WES).

In addition, there is accumulating evidence for PVs contributing to schwannomatosis combined with other conditions has been set by the case for families affected by more than one condition in which a particular variant is observed to co-segregate with disease, such as the previously described variant in *DGCR8* (Rivera et al., 2020) or for families affected by *CDKN2A*-associated melanoma, who also have an increased risk for other cancers present (Goldstein et al., 2006). This is also the case for people affected by melanoma along with nervous system tumours due to deletion of both *CDKN2A* and *CDKN2B*, with suggestions that inactivation of *CDKN2A/B* genes might be responsible for the melanoma phenotype, while loss of adjacent genes might contribute to the development of other neoplasms (Chan et al., 2017). The presence of schwannomas in our melanoma patient and the retention of the single base duplication in *CDKN2A* in tumour DNA suggest that inactivation of *p14ARF* and *p16ink4a*, may be enough for schwannoma formation. The presence of additional factors contributing to risk for both conditions has also been explored in previous studies. One intriguing possibility is the potential effect of non-coding elements on the observed phenotype. One of these elements, a long non-coding RNA (*CDKN2B-AS1*) spanning the two exons of *CDKN2B*, was discovered by a study aimed to determine the size of a 9p21 deletion in a large family affected by melanoma-astrocytoma syndrome (Pasmant et al., 2007). The authors suggest, *p14ARF* and *CDKN2B-AS1* might share a promoter, a fact supported by their discovery of a significant correlation in transcript levels of *CDKN2B-AS1* with those of *p14ARF*, *p16INK4a* and *CDKN2B* in healthy tissue as well as breast tumour samples and NF1-associated tumour samples.

Exploration of the possible interactions of potential candidate genes for schwannomatosis with the known causative genes, might provide some insight into the type and location of

novel PVs in cases where no variants in *SMARCB1*, *LZTR1* or *NF2* have been identified. Furthermore, functional characterisation of known causative variants for schwannomatosis will undoubtedly advance our understanding of potentially new mechanisms of disease in schwannomatosis, particularly for variants located in non-coding regions of both *SMARCB1* and *LZTR1*. This in turn might lead to elucidation of important correlations of these genes with other loci, and ultimately to an increased ability for accurate diagnosis and classification of schwannomatosis cases based on their clinical and molecular features.

Acknowledgements

This work was supported by USAMRAA CDMRP Neurofibromatosis Research Program, Investigator-Initiated Research Award (W81XWH1910334). DGE and MJS are also supported by the Manchester National Institute for Health Research (NIHR) Biomedical Research Centre (IS-BRC-1215-20007).

Conflict of interest

The authors have no conflict of interest to declare

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

An entry has been made for the *CDKN2A* NM_000077.4:c.158dup variant in the LOVD database and can be found here:

https://databases.lovd.nl/shared/variants/0000839293#00004876

Web Resources

gnomAD:

https://gnomad.broadinstitute.org/

DECIPHER:

https://www.deciphergenomics.org/

Ensembl:

https://www.ensembl.org/index.html

Frequency Filter:

http://cardiodb.org/allelefrequencyapp/

SpliceAI

https://spliceailookup.broadinstitute.org/

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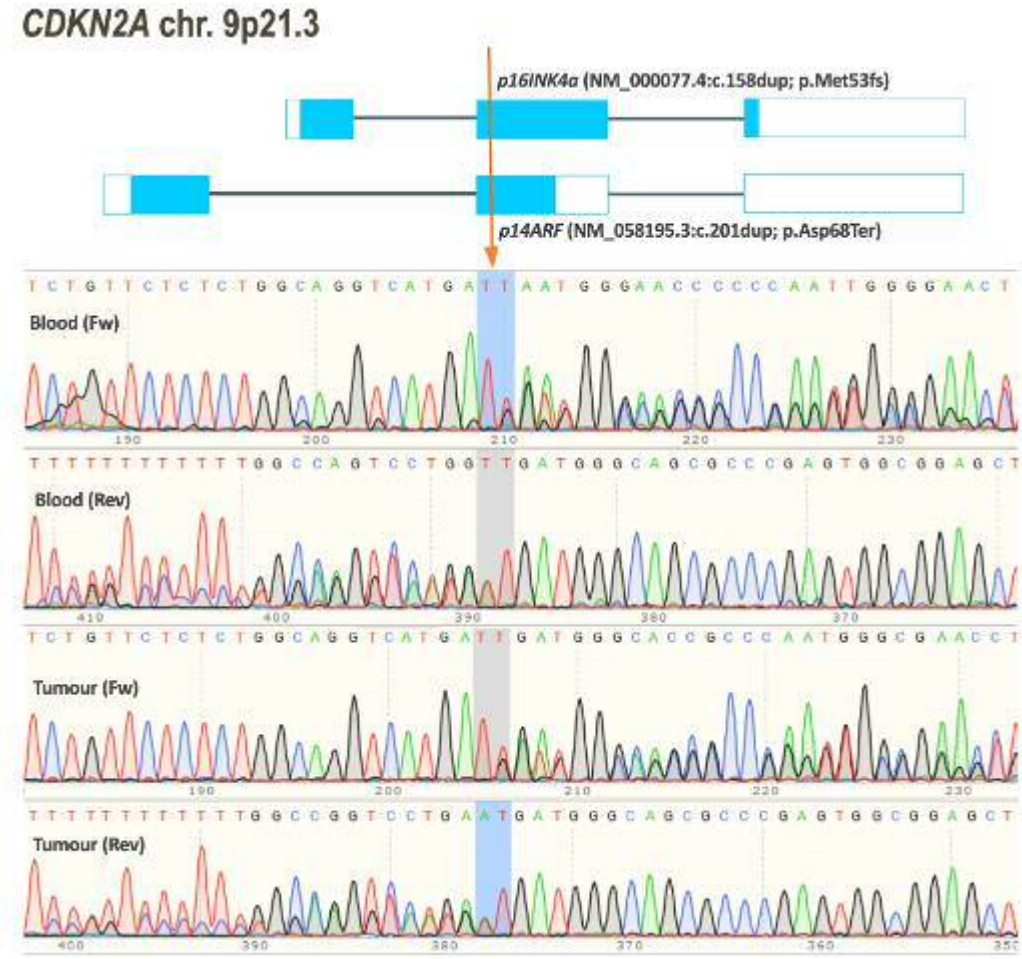

Figure 1. Duplication in CDKN2A in a schwannomatosis patient also diagnosed with melanoma.

Schematic representation of both isoforms of *CDKN2A*, indicating the position of a single base pair (T) duplication that results in a frame shift for both proteins, p16INK4a (NM_000077.4:c.158dup; p.Met53fs) and p14ARF (NM_058195.3:c.201dup; p.Asp68Ter). The duplication was identified by bidirectional sequencing of a lymphocyte derived DNA sample from a schwannomatosis patient who was also diagnosed with melanoma. Additionally, a

schwannoma sample was available for sequencing analysis, which confirmed the presence of this variant in the tumour.