Improving *SMARCA4* variant classification in the context of small cell carcinoma of the ovary, hypercalcemic type

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Abstract

Purpose: *SMARCA4* germline pathogenic variants (GPVs) are associated with small-cell carcinoma of the ovary, hypercalcemic type (SCCOHT), atypical rhabdoid tumor (ATRT), and malignant rhabdoid tumor (MRT). SCCOHT is a rare, highly aggressive monogenic disorder occurring in young females contributing to ~0.01% of all ovarian cancers, with a mean age of diagnosis of 24 years. SCCOHT presents significant challenges with clinical management and lack of extensive research due to its rarity, aggressive nature, limited treatment options and poor prognosis. *SMARCA4* encodes a critical component of the SWI/SNF chromatin remodeling complex, which contributes to the maintenance of proper gene expression through alteration of the chromatin architecture. *SMARCA4* is now included in many cancer susceptibility gene panel tests but it is important to note that GPVs in *SMARCA4* and related genes can also cause Coffin-Siris Syndrome (CSS), a neurodevelopmental disorder. GPVs causing CSS are generally missense, whereas pathogenic variants causing SCCOHT are usually nonsense or frameshift. However, individuals with SCCOHT who harbor missense pathogenic variants and CSS patients with predicted truncating variants have been observed. It is not known why some *SMARCA4* missense variants cause intellectual disability while others cause cancer. Therefore, correctly classifying these variants is crucial for determining whether carriers are at risk for cancer. Currently, the absence of gene-specific classification criteria for *SMARCA4* results in misclassification of many variants. This misclassification occurs because of the lack of validated functional data and a scarcity of clinical data due to the disease's rarity, resulting in problems and concerns with classification, particularly the classification of missense variations. This leads to challenges with clinical management, especially as the main risk-reducing intervention for unaffected women with a GPV in *SMARCA4* is preventive oophorectomy. Our study aimed to create *SMARCA4*-specific variant classification criteria for SCCOHT, leveraging a database of 2870 variants collected from diverse sources. Additionally,

we performed functional studies to assess the pathogenicity of certain variants by utilizing cell-based assays.

Methods: We collected variant data from 9 sources (n = 2870 variants) and integrated available genomic and expression data, our own laboratory data, and patient phenotypic profiles to develop refined ACMG/AMP classification criteria specific to *SMARCA4* and its associated cancers. Functional studies performed include DNA methylation, cell viability and protein expression assays using a SCCOHT cell line (SCCOHT-1) expressing benign, missense, and CSS-related *SMARCA4* variants.

Results: We established refined *SMARCA4*-specific criteria and confidently reclassified 69 non-truncating *SMARCA4* variants, providing clinically important information for heterozygotes of these variants. Additionally, we classified 64 truncating *SMARCA4* variants seen in 73 previously unreported patients with SCCOHT, MRT, or ATRT.

Conclusion: Results from this thesis and our updated gene-specific classification criteria will aid in better classification of *SMARCA4* variants and will minimize the number of Variants of Uncertain Significance (VUSs) reported. Improved classification of *SMARCA4* variants will result in more effective genetic testing and counselling and will deepen our understanding of the mechanisms by which missense pathogenic variants in *SMARCA4* lead to SCCOHT or CSS. By distinguising pathogenic variants for SCCOHT, we can more accurately investigate how these variants distrupt SMARCA4 function and contribute to cancer and neurodevelopmental disorder. This appoach will shed light on the underlying mechanisms driving these cancers, which will guide the development of better targeted therapies. Future work on functional studies validating the impact of specific variants on SMARCA4 function and tumor development will strengthen the evidence for variant pathogenicity and will generate preliminary data for studying the etiology of SCCOHT development.

Résumé

Objectif : Les variants pathogènes germinaux (VPGs) du gène *SMARCA4* sont associés au cancer de l'ovaire à petites cellules de type hypercalcémique (SCCOHT), aux tumeurs rhabdoïdes atypiques (ATRT) et aux tumeurs rhabdoïdes malignes (MRT). SCCOHT est une maladie monogénique rare et très agressive qui survient chez les jeunes femmes et qui représente environ 0,01 % de tous les cancers de l'ovaire, l'âge moyen du diagnostic étant de 24 ans. SCCOHT présente des défis importants en termes de gestion clinique dû au manque de recherches approfondies en raison de sa rareté et agressivité, de ses options thérapeutiques limitées et de son mauvais pronostic. *SMARCA4* code pour un composant essentiel du complexe de remodelage de la chromatine SWI/SNF, qui contribue au maintien d'une expression génétique correcte par l'altération de l'architecture de la chromatine. *SMARCA4* est désormais inclus dans de nombreux tests de susceptibilité génétique au cancer, mais il est important de noter que les VPGs dans *SMARCA4* et les gènes apparentés peuvent également causer le syndrome de Coffin-Siris (CSS), un trouble du développement neurologique. Les VPGs à l'origine du CSS sont généralement faux-sens, tandis que ceux à l'origine du SCCOHT sont non-sens ou décalage du cadre de lecture. Cependant, nous avons observé des individus atteints de SCCOHT qui portent des variants pathogènes faux-sens et des patients atteints de CSS avec des variants prédits tronquants. Nous ignorons pourquoi certains variants faux-sens de *SMARCA4* provoquent une déficience intellectuelle alors que d'autres sont à l'origine d'un cancer. Par conséquent, une classification exacte de ces variants est cruciale pour déterminer si les porteurs présentent un risque de cancer. Présentement, l'absence de critères de classification spécifiques au gène *SMARCA4* entraîne une classification erronée de nombreux variants, en particulier celle des variations faux-sens. Cela s'explique par l'absence de données fonctionnelles fiables et la rareté des données cliniques. Ceci entraîne des difficultés dans la gestion clinique, d'autant plus que la principale intervention de réduction des risques pour les

femmes non affectées ayant un VPGs dans *SMARCA4* est l'ovariectomie préventive. Notre étude visait à créer des critères de classification des variants spécifiques à *SMARCA4* et au SCCOHT, ATRT et MRT, en tirant parti d'une base de données de 2870 variants recueillis auprès de diverses sources. En outre, nous avons réalisé des études fonctionnelles pour évaluer la pathogénicité de certains variants en utilisant des tests cellulaires.

Méthodes : Nous avons recueilli des données sur les variants auprès de 9 sources (n = 2870 variants) et intégré les données génomiques et d'expression disponibles, nos propres données de laboratoire et les profils phénotypiques des patients afin de développer des critères de classification ACMG/AMP affinés spécifiques à *SMARCA4* et aux cancers qui lui sont associés. Les études fonctionnelles comprennent des essais de méthylation de l'ADN, de viabilité cellulaire et d'expression des protéines à l'aide d'une lignée cellulaire SCCOHT (SCCOHT-1) exprimant des variants *SMARCA4* bénins, faux-sens et liés au CSS.

Résultats : Nous avons établi des critères affinés spécifiques à *SMARCA4* et reclassé 69 variants *SMARCA4* non tronqués, fournissant des informations cliniques importantes pour les hétérozygotes de ces variants. Nous avons aussi classé 64 variants tronqués de *SMARCA4* observés chez 73 patients qui n'ont pas été publiés ailleurs.

Conclusion : Cette thèse contribuera à une meilleure classification des variants du gène *SMARCA4* et réduira le nombre de variants de signification incertaine signalés. Ceci permettra d'améliorer l'efficacité des tests et des conseils génétiques et d'approfondir notre compréhension des mécanismes par lesquels les variants pathogènes faux-sens de *SMARCA4* conduisent au SCCOHT ou au CSS. En distinguant les variants pathogènes de SCCOHT, nous pouvons étudier plus précisément comment ces variants perturbent la fonction de SMARCA4 et contribuent au cancer et aux troubles du développement neurologique. Cette approche permettra de mettre en lumière les mécanismes sous-jacents de ces ma, ce qui guidera le développement de meilleures thérapies ciblées. De futures études fonctionnelles validant

l'impact de variants spécifiques sur la fonction de SMARCA4 et le développement tumoral renforceront les preuves de la pathogénicité des variants et généreront des données préliminaires pour étudier l'étiologie du développement du SCCOHT.

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List of Abbreviations

GPV	Germline Pathogenic Variant
SCCOHT	Small Cell Carcinoma of the Ovary, Hypercalcemic Type
ATRT	Atypical/Typical Teratoid Tumor
MRT	Malignant Rhabdoid Tumor
CSS	Coffin-Siris Syndrome
SWI/SNF	SWItch/Sucrose non-fermentable
BAF	BRG1/BRM-Associated Factor
PBAF	Polybromo-Associated BAF
ncBAF	Non-Canonical BAF
BRG1	Brahma Related Gene 1
BRM	Brahma
ID	Intellectual Disability
RTPS2	Rhabdoid Tumor Predisposition Syndrome Type 2
SCCOPT	Small Cell Carcinoma of the Ovary, Pulmonary Type
LOH	Loss-of-heterozygosity
LoF	Loss-of-function
IHC	Immunohistochemistry
pLoF	Predicted Loss-of-function
pLI	Probability of loss-of-function intolerance
O/E	Observed/expected
HRT	hormone replacement therapy
HDC-aSCR	high-dose chemotherapy with autologous stem cell rescue
EZH2	Enhancer Of Zeste 2 Polycomb Repressive Complex 2 Subunit

HDAC	Histone deacetylase
CDK4/6	Cyclin-dependent kinase 4/6
NSCLC	Non-Small Cell Lung Carcinoma
ECRT	extracranial rhabdoid tumor
ACMG	American College of Medical Genetics and Genomics
AMP	American Association of Molecular Pathology
VUS	Variant of Uncertain Significance
LP	Likely Pathogenic
P	Pathogenic
LB	Likely Benign
B	Benign
VCEP	Variant Curation Expert Pannel
LCL	Lymphoblastoid Cell Line
PV	Pathogenic Variant
SVI	Sequence Variant Interpretation
NMD	Nonsense-mediated decay
ER	Endoplasmic reticulum
FBS	Fetal Bovine Serum

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Contribution of authors

Marie Loncol analyzed the data and wrote the thesis. Nancy Hamel and William D. Foulkes provided substantial feedback and editorial help on the thesis. Marie Loncol, Leora Witkowski, William D. Foulkes, Jialing Jiang and Sidong Huang designed the experiments. Leora Witkowski and William D. Foulkes provided substantial direction on the project. Céline Domecq provided guidance throughout all wet lab projects. Jialing Jiang provided guidance throughout the development and execution of functional assays. José Camacho Valenzuela and Ana Castillo Orozco provided help with the RNA sequencing analysis.

1. Introduction

1.1. SWItch/Sucrose Non-Fermentable (SWI/SNF) chromatin-remodeling complex

DNA is packaged into chromatin, containing histone octamers around which the DNA is wrapped. Chromatin is further regulated by epigenetic modifications and ATP-dependent chromatin remodeling complexes, including the SWItch/Sucrose Non-Fermentable (SWI/SNF) chromatin-remodeling complex.[1] There are three primary types of mammalian SWI/SNF complexes, namely the BRG1/BRM-associated factor (BAF) complex, the polybromo-associated BAF (PBAF) complex and the non-canonical BAF (ncBAF) complex.[2] The BAF SWI/SNF complex is composed of several subunits, including the ATPase subunits SMARCA4 (BRG1) or SMARCA2 (BRM), which are mutually exclusive and share approximately 72% of coding sequence.[3] Other subunits include SMARCB1 (SNF5), SMARCC1 (BAF155) and SMARCE1 (BAF57). The catalytic subunits are characterized by an ATPase domain. The SWI/SNF complex is crucial for the regulation of gene expression, as it specificially alters chromatin structure by utilizing the energy from ATP hydrolysis to disassembles nucleosomes and creating nucleosome-deficient regions of DNA (**Figure 1**).[1] This alteration of DNA accessibility to transcription factors and other regulatory elements allows the SWI/SNF complex to both activate and suppress gene expression.[1] Notably, pathogenic variants in gene encoding these subunits of these complexes have been found in 20% of all cancers and are also found in persons with intellectual disability (ID).[2] As many of the SWI/SNF subunits act as tumor suppressors, the loss-of-function of these subunits can drive oncogenic phenotypes.[2]

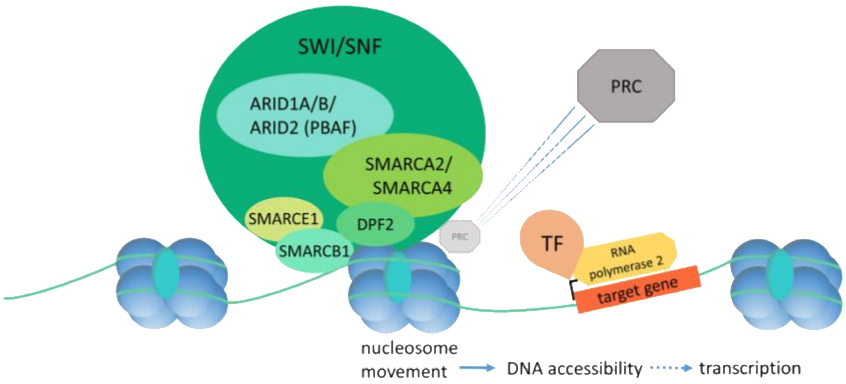


Figure 1. The SWI/SNF BAF complex. Retrieved from Bögershausen and Wollnik., 2018.[4] © 2018 Bögershausen and Wollnik. Distributed under the terms of the Creative Commons Attribution License (CC BY).

1.2. SMARCA4

The *SMARCA4* gene encodes the SMARCA4 transcriptional activator protein, also known as BRG1, which is an ATPase subunit of the SWI/SNF chromatin remodeling complex. Its key function is attributed to its ATPase domain, which moves nucleosomes and alters chromatin structure using the energy from ATP hydrolysis, allowing transcriptional regulators or repressors to bind to DNA (**Figure 2**).[5,6] These processes convey the role of the SWI/SNF complex, particularly SMARCA4, in various cellular functions, including DNA replication, growth and division of cells, and DNA repair, functioning as a tumor suppressor.[5] *SMARCA4* comprises 35 or 36 exons, depending on the transcript, and is found on chromosome 19p.[5] *SMARCA4* is aberrant in approximately 5–7% of all human malignancies, including small-cell carcinoma of the ovary, hypercalcemic type (SCCOHT), thoracic sarcomatoid tumors and malignant rhabdoid cancers, and cause rhabdoid tumor predisposition syndrome type 2 (RTPS2) when mutated in the germline.[5]

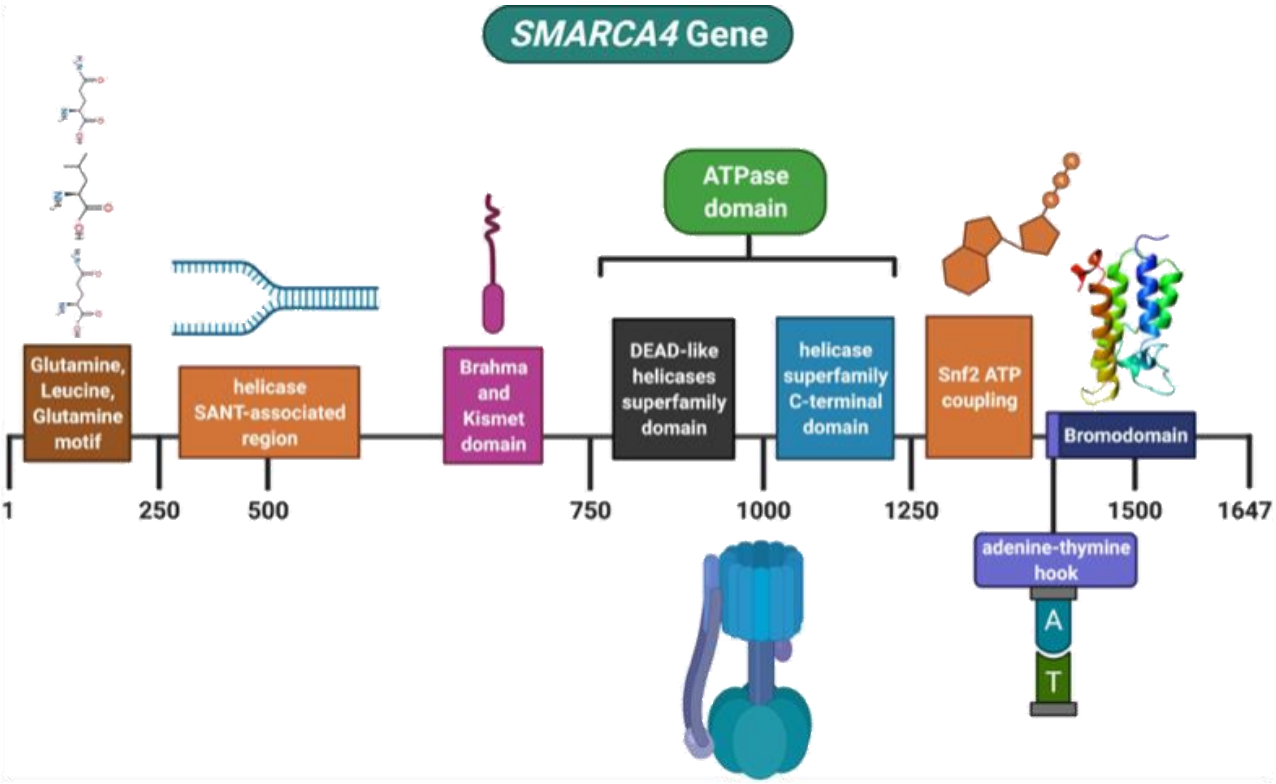


Figure 2. Structure of the SMARCA4 gene. Permissions to re-use figure obtained from the publisher. Retrieved from Mardinian et al., 2021.[5]

1.3. Small-cell carcinoma of the ovary, hypercalcemic type (SCCOHT)

Small-cell ovarian cancer is rare and aggressive form of ovarian cancer, which is categorized into two types: the hypercalcemic type and the pulmonary type.[7] They have distinct differences in terms of clinical presentation, molecular characteristics, and pathological features, discussed below.

The pulmonary type, known as small-cell carcinoma of the ovary, pulmonary type (SCCOPT), is less common of the two disorders, usually affecting older women, with a mean age of diagnosis of 59 years.[8] Although having similar symptoms as the hypercalcemic type, such as abdominal pain, SCCOPT is not associated with hypercalcemia and more importantly is not caused by *SMARCA4* variants. Its molecular background has not yet been elucidated.

Moreover, both disorders are composed of small tumor cells, however, compared to SCCOHT, SCCOPT cells have a rosette-like appearance and are often immunopositive for neuroendocrine markers such as chromogranin.[8] Furthermore, while SCCOHT is often resistant to chemotherapy and radiotherapy, SCCOPT demonstrates a responsiveness to chemotherapy. Patients are often treated with similar treatment used for small cell lung carcinoma.[7]

The hypercalcemic type, known as small-cell carcinoma of the ovary, hypercalcemic type (SCCOHT), is a rare and aggressive, predominantly monogenic disorder with very occasional genetic heterogeneity,[9,10] that usually occurs in an age range that encompasses adolescent to young adult women, with a mean age of diagnosis of 24 years. The age range for this disease however, can vary from infants to women in their 50s.[6] Less than 600 cases are reported in literature, making this disease rare in incidence.[11] The initial description of this disease was provided by Robert E. Scully in 1979, who characterized it as a structure resembling a follicle with a diffuse arrangement of cells. These cells had small, hyperchromatic nuclei with scant cytoplasm and exhibited high levels of mitotic activity (**Figure 3**).[12] About two-thirds of the patients have hypercalcemia. [6] SCCOHT tumor cells are also said to look "rhabdoid", as they have a similar apparency to atypical teratoid/rhabdoid tumors (ATRTs) and malignant rhabdoid tumors (MRTs), with eosinophilic cytoplasm being abundantly present (**Figure 4**).[13] Moreover, DNA methylation profiling shows robust epigenetic correlation between SCCOHT and ATRT, further supporting this statement.[14]

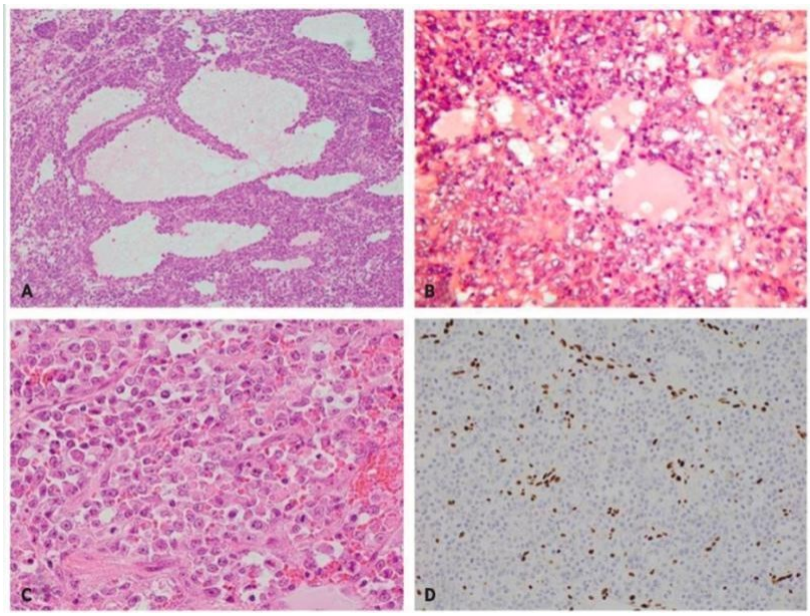


Figure 3. Morphology and immunohistochemistry of SCCOHT. SCCOHT is composed of predominantly diffuse arrangement of cells with follicle-like structures (A). On higher power, the tumor cells have hyperchromatic nuclei and scant cytoplasm (B). Large cell variant of SCCOHT composed of tumor cells with abundant eosinophilic cytoplasm (C). There is loss of nuclear immunoreactivity with SMARCA4 (BRG1) with a positive internal control in the form of nuclear staining of endothelial cells (D).Permissions to re-use figure obtained from the publisher. Retrieved from Tischkowitz et al., 2020.[6]

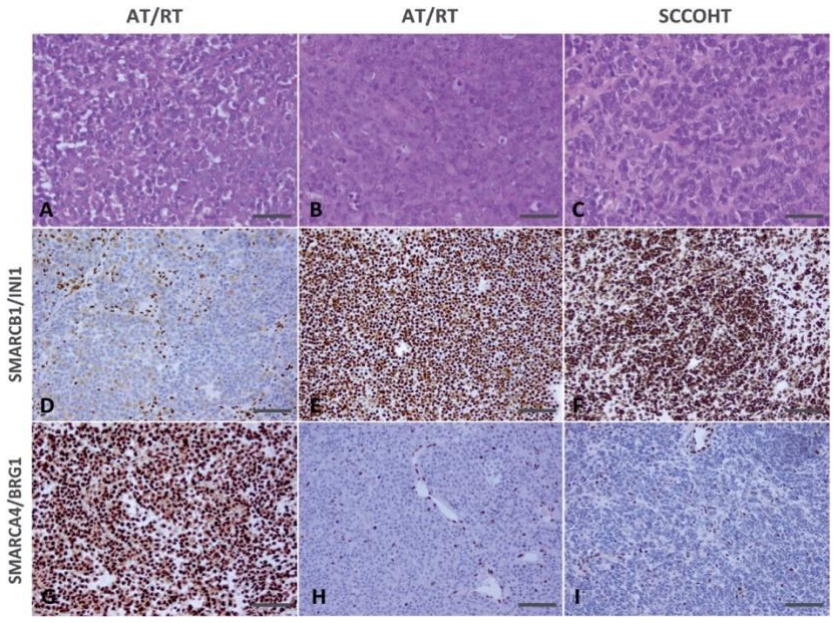


Figure 4. Morphological and immunohistochemical comparisons of AT/RTs and SCCOHT. SMARCB1-deficient ATRT (left), SMARCA4-deficient ATRT (middle) and SCCOHT (right). Permissions to re-use figure obtained from the publisher. Retrieved from Foulkes et al., 2014.[13]

In 2014, four research teams almost simultaneously uncovered the fact that SCCOHT is distinguished by both germline and somatic deleterious variants in the *SMARCA4* gene.[15,16,17,18] In these instances, SMARCA4 operates as a suppressor of tumorigenesis. Pathogenic *SMARCA4* variants often lead to loss of SMARCA4 expression, although not all cases show absence of statining by immunohistochemistry.[19] The inactivation or loss of SMARCA4 through two hits likely results in transcriptional and epigenetic dysregulation, but the precise mechanism by which this drives tumorigenesis is not yet understood. These two hits are usually comprised of truncating variants, frameshift variants, deletions, or splice variants.[9] Certain pathogenic missense variants exhibit retained expression, some cases show loss of heterozygosity (LOH), whereas others have second hits predicted to be truncating loss-of-function (LoF) variants.[19] SMARCA4 retention complicates the understanding of its role in tumor development. The SMARCA4 antibody is extremely helpful with the diagnosis of SCCOHT, since loss of SMARCA4 nuclear immunoreactivity occurs in most cases, though, as discussed, occasional tumors exhibit SMARCA4 retention. Moreover, extremely rare reports of GPVs in SMARCB1 causing SCCOHT exist, with retention of SMARCA4.[9,10] Therefore, one should be careful with using only IHC for diagnosis, and tumor sequencing should be considered to confirm diagnosis. Diagnosis of SCCOHT may furthermore be difficult, due to morphological overlap with an extensive variety of differential diagnoses of various neoplasms.

SMARCA4 LoF variants are valuable for the diagnosis of SCCOHT. The gnomAD database (https://gnomad.broadinstitute.org, v4.1, this version does not include any enrichment for cancer cases) reports that predicted LoF (pLOF) variants in *SMARCA4* are extremely rare, giving *SMARCA4* a high probability of loss-of-function intolerance (pLI). pLI is a metric used in genetics to assess how intolerant a gene is to LoF variants. It is derived from population data and is a measure of how frequently LoF variants occur in a given gene within a population, ranging from 0 (suggesting that the gene is tolerant to LoF variants, meaning these variants occur more frequently in the population without severe consequences) to 1 (indicating that the gene is highly intolerant to LoF variants, meaning such variants are very rarely observed in the population).[20] *SMARCA4* has a pLI with a score of 1.0, further emphasizing the gene's high intolerance to LoF variants.[21] Additionally, the gnomAD database reports the observed/expected (O/E) score, indicated the ratio of the observed/expected variants in a gene, and therefore indicates how tolerant a gene is to a certain class of variants.[20] Low O/E value suggests that a gene is under stronger selection for that class of variants than a gene with a higher O/E value. *SMARCA4* has an O/E score of 0.17 for pLoF. This data suggests that LoF in this gene are likely deleterious. The low allele frequency in different populations suggests that these variants are either acquired de novo or inherited from the father, as women carrying pathogenic LoF variants typically do not survive, leading to their absence in population frequency data.

1.4. Management of SCCOHT

1.4.1. Prevention

Current strategies of prevention of SCCOHT are limited to surgical prevention. Genetic testing and counseling for patients and their family members is crucial to identify germline *SMARCA4* variants and assess the risk of developing the disease. Persons found to carry *SMARCA4*

variants might consider preventive strategies.[22] They should consider preventive bilateral oophorectomy which is currently the main risk-reducing procedure for unaffected women carrying a pathogenic variant and which has a significant impact on life. Optimal timing of this procedure is uncertain due to lack of penetrance data.[22] The first reported case of preventive oophorectomy was performed on a 33-year old unaffected woman, carrying a pathogenic *SMARCA4* variant, whose two adult sisters had died of SCCOHT.[23] Since then, case reports of preventive bilateral oophorectomy performed in children have been published; Pejovic et al. report a case of a 13-year old girl.[24] Both her mother and aunt were diagnosed with SCCOHT, and died at age 26 and 27 respectively. The 13-year-old girl underwent hormone replacement therapy (HRT) with estrogen and progesterone following her surgery. There are currently no official recommendations regarding the age of preventive bilateral oophorectomy. Experts recommend bilateral oophorectomy for female carriers of deleterious pathogenic *SMARCA4* variants after completion of puberty. However, some may argue that due the extremely poor outcome of the disease and due to the lack of early diagnosis and prevention, preventive bilateral oophorectomy may be considered for underaged females.[25] Nevertheless, this decision necessitates a thorough genetic counseling process, evaluating the risks and benefits of this procedure and the timing of it. Benefits include the avoidance of a devastating cancer (given the lack of effective treatment) and limiting the psychological burden of living with a high risk of cancer. Given hormone replacement therapy (HRT) is available, this may be the preferred choice for some younger females. Fertility preservation might be possible. On the other hand, the risks include the likely loss of fertility, need of long-term management of hormone therapy, the possibility of depression and anxiety, concerns about premature aging, and possibly a heightened risk of breast cancer associated with prolonged HRT. Freezing ovarian tissue for fertility is experimental and given the cell of origin of SCCOHT may be germ cells, could be

itself risky.[24] [26,27] As discussed, many bioethical considerations arise in this procedure and the use of HRT, for a rare, aggressive disease, with presumed high penetrace.[28]

1.4.2. Early Diagnosis

There is currently no effective method for the early diagnosis of SCCOHT. While imaging techniques have been considered as potential diagnostic tools, their efficacy in early detection is limited.[6]

The treatment of SCCOHT typically involves multimodal therapy including high-dose chemotherapy followed by autologous stem cell transplantation (HDC-aSCR), offering the best chance for long-term survival by preventing recurrence despite overall relapse rates being 65%.[22] Patients ideally should have a complete chemotherapy response before HDC-aSCR. Surgery alone is inadequate. However, surgical intervention followed by cisplatin- and platinum-based chemotherapy are commonly used. Radiation may be considered, although the role of radiotherapy remains unclear. In young patients with unilateral disease, fertility-conserving surgery may be considered to preserve reproductive potential. Patients with stage I disease have an estimated long-term survival rate of 33%, while the overall survival rate ranges from 10% to 20%.[6] There is thus an urgent need to identify novel therapeutic strategies to treat SCCOHT.

1.4.3. Novel Therapeutic Approaches

Given the limited treatment options and poor prognosis of the disease, novel therapeutic approaches are currently being explored. Recent studies have identified promising targetable vulnerabilities associated with SCCOHT. These include EZH2 inhibitors, HDAC inhibitors, ponatinib, CDK4/6 inhibitors, and arginine depletion therapy with ADI-PEG20.

Wang et al. have showed that the methyltransferase EZH2 could suppress growth of SCCOHT cells in preclinical models.[29] This potential therapeutic target is at the early stages of clinical studies. A year later, this group also showed that HDAC inhibitors, a class of anticancer drugs regulating gene expression through interrupting the deacetylation of histones and nonhistone proteins, suppress growth of SCCOHT cells.[30] They further showed that upon combination with EZH2 inhibitors, there is a synergistic effect in growth suppression of SCCOHT cells and xenograft tumors.[30] That year, Lang et al. also discovered that ponatinib, a tyrosine kinase inhibitor, could be antitumor in SCCOHT through inhibition of multiple kinases both in vitro and in vivo preclinical model.[31]

Moreover, Xue et al., showed that SMARCA4-deficient SCCOHT cells exhibit cyclin D1 deficiency.[32] In fact, they showed that SMARCA4 loss in SCCOHT leads to reduced cyclin D1 expression, which limits CDK4/6 kinase activity and renders the cells susceptible to CDK4/6 inhibitors, both in vitro and in vivo. Therefore, the use of CDK4/6 inhibitors is proposed as targeted therapy. Furthermore, since SMARCA4 and SMARCA2 are mutually exclusive ATPase subunits of the SWI/SNF complex, SMARCA2 has been suggested as potent target for the treatment of SCCOHT.[33,34,35] Cells deficient in either of these subunits depend on the remaining one for survival.[36] SCCOHT tumors, however, do not express SMARCA2, and no variants in this gene have been identified, implicating epigenetic silencing or degradation during tumourigenesis as the underlying mechanism for the absence of SMARCA2 protein.[34] Overexpression of either SMARCA4 or SMARCA2 in SMARCA4-deficient SCCOHT cell lines inhibits cell growth, suggesting that loss SMARCA2 is required for tumor growth.[34] Furthermore, it has been documented that the inhibition of SMARCA2 is synthetically lethal in combination with the loss of SMARCA4 in non-small cell lung carcinoma (NSCLC) cells.[32] Notably, SMARCA2 expression can be restored in SCCOHT cell lines with the use of HDAC inhibitors, which strongly suppresses cell growth, indicating a potential treatment for

SCCOHT.[34,30] Interestingly, the reintroduction of SMARCA4 in SCCOHT cell lines did not result in the restoration of SMARCA2, suggesting that SMARCA4 alone is not sufficient for the re-expression of SMARCA2.[37,38,39] Further studies have showed that SMARCA4/2 deficiency impairs chemotherapy-induced apoptotic responses in ovarian and lung cancers by altering ER to mitochondria Ca2+ flux.[33] Specifically, SMARCA4/2 loss restricts ITPR3 expression, which is essential for Ca2+ transfer required for apoptosis induction. Reactivating SMARCA2 with HDAC inhibitors was shown to enhance chemotherapy response in SMARCA4/2-deficient cancer cells by stimulating ITPR3 expression. This discovery provides a potential therapeutic strategy to improve chemotherapy efficacy in patients with SMARCA4/2-deficient cancers.

Moreover, Ji et al. showed that arginine depletion therapy with ADI-PEG20 was effective in controlling SCCOHT tumor growth in cell lines and xenograft models. Therefore, ADI-PEG20 could serve as promising therapy.[40]

Lastly, Zhu et al. showed the use of alanine supplementation as a promising therapy by demonstrating that alanine supplementation can selectively target SMARCA4/2-deficient cancer cells by exploiting their increased dependency on glutamine for survival. [36]

1.5. Role of SMARCA4 in Rhabdoid Predisposition Syndrome Type 2 (RPST2)

Rhabdoid predisposition syndrome type 2 (RTPS2) is associated with the development of rhabdoid tumors, including atypical teratoid/rhabdoid tumors (ATRTs) and malignant rhabdoid tumors (MRTs). ATRTs are aggressive malignant rhabdoid brain tumors occurring in infants, whereas MRTs most commonly occur in the kidney.[41] MRTs are also referred to as extracranial rhabdoid tumors (ECRTs). Germline pathogenic variants in *SMARCB1*, another core member of the chromatin remodeling complex, are responsible for >95% of cases of ATRTs and

MRTs.[42] Initially, it was thought that SMARCB1 immunohistochemistry could unequivocally identify all ATRTs and MRTs. However, in 2010, Schneppenheim et al. identified inactivation of SMARCA4 due to a *SMARCA4* germline variant and LOH in tumor cells of two sisters with rhabdoid tumors lacking *SMARCB1* variants.[43] In addition to *SMARCB1*, *SMARCA4* GPVs have then been identified as the cause of RTPS2. Subsequently, GPVs in *SMARCA4* were identified as the cause of SCCOHT in 2014. The storyline was coherent, given that these tumors closely resemble ATRTs and MRTs. SCCOHT shares similarities with rhabdoid tumors on pathological, clinical, and molecular levels, and some argue that it represents malignant rhabdoid tumor of the ovary.[22] It has been reported that *SMARCA4*-mutated ATRTs are associated with a worse prognosis.[44] Since the establishment of the association with these cancers, *SMARCA4* has been added to multi-gene hereditary cancer testing panels in several commercial and academic laboratories, particularly ovarian cancer panels.

1.6. Role of SMARCA4 in Coffin-Siris Syndrome

Coffin-Siris syndrome (CSS) is a neurodevelopmental disorder, characterized by intellectual disability, microcephaly, coarse facial features, sparse scalp hair, growth deficiency, and hypoplastic nail of the fifth finger and/or toe.[45] 12 genes have been identified as being associated with CSS, namely, ARID1B, ARID1A, ARID2, DPF2, PHF6, SMARCB1, SMARCA2, SMARC4, SMARCC2, SMARCE1, SOX4 and SOX11, with ARID1B being the most common.[46] However, pathogenic variants in the *SMARCA4* gene can also cause CSS (11% of all cases).[47] It has been suggested that CSS patients have an increased risk for cancer, but it has never been proven.[47] Proof of an increased cancer risk in CSS patients would require epidemiological studies demonstrating a significant higher incidence of cancer in individuals with CSS compared to the general population, and functional studies showing that CSS-related *SMARCA4* variants drive oncogenesis similarly to SCCOHT-related variants. However,

conducting large-scale epidemiological studies is challenging due to the rarity of the disease, and no functional studies currently exist. However, Errichiello et al reported a case of a 15-year-old CSS patient who developed SCCOHT at 13 years old, supporting evidence that chromatin remodeling factors may simultaneously cause developmental disorder and cancer.[47]

1.7. Comparing *SMARCA4* alteration in Rhabdoid Tumor Predisposition Syndrome Type 2 and Coffin-Siris Syndrome

SMARCA4 pathogenic variants can cause cancer and neurodevelopmental disorders. However, both can generally be distinguished since the GPVs causing CSS are typically missense or small in-frame deletions, non-truncating variants whereas those causing RTPS2 are generally nonsense or frameshift, truncating LoF variants. However, the opposite has been observed, with truncating frameshift variants leading to CSS and missense non-truncating variants causing RTPS2.[15,44,47-49] It not known why some *SMARCA4* pathogenic variants cause intellectual disability while others cause cancer. Nevertheless, SCCOHT presents intriguing properties that could be further explored in CSS. In SCCOHT, as discussed above, loss of SMARCA4 function leads to critically low levels of cyclin D1, which limits the activity of cyclin-dependent kinase 4/6 (CDK4/6), making them sensitive to FDA-approved CDK4/6 inhibitors.[32] Cyclin D1 is a vital protein that plays a crucial role in regulating the cell cycle by forming complexes with CDK4/6, thereby promoting cell cycle progression. In the absence of SMARCA4, SMARCA2 compensates by regulating cyclin D1 expression. The loss of SMARCA4/2 together is specific to SCCOHT and non-small cell lung cancer (NSCLC),[50] and impacts the cell cycle via cyclin D1.[32] Given the central role of *SMARCA4* variants in the pathogenesis of SCCOHT and their implications for preventive intervention decisions and targeted therapy, correct classification of these variants is thus crucial to determine who is truly at risk for cancer.

1.8. ACMG/AMP classification criteria

In 2015, the American College of Medical Genetics and Genomics (ACMG) and Association for Molecular Pathology (AMP) established a classification system for sequence variants, which defined criteria with codes that addressed types of variant evidence. It categorizes variants into five classes: pathogenic, likely pathogenic, uncertain significance, likely benign, and benign.[51] Each criterion is arranged according to the type of evidence, including population data, computational and predictive data, functional data, segregation data, de novo data, allelic data, and other databases or other date, and strength of the criteria for a benign or pathogenic classification in **Figure 5**, which displays the evidence categories and levels from the ACMG/AMP classification. These evidence types are then used to assess a variant according to specific criteria. The criteria are weighted as very strong (Pathogenic very strong: PVS1), strong (Pathogenic strong: PS1-PS4), moderate (Pathogenic moderate: PM1-PM6), and supporting (Pathogenic supporting: PP1-PP5) for pathogenicity, and similarly weighted for benign criteria (Benign stand alone: BA1, Benign strong: BS1-BS4, Benign supporting: BP1-BP7). Gene-specific classification criteria exist, adapting these criteria for the specific evidence and mechanisms of action of a certain gene in a disease.

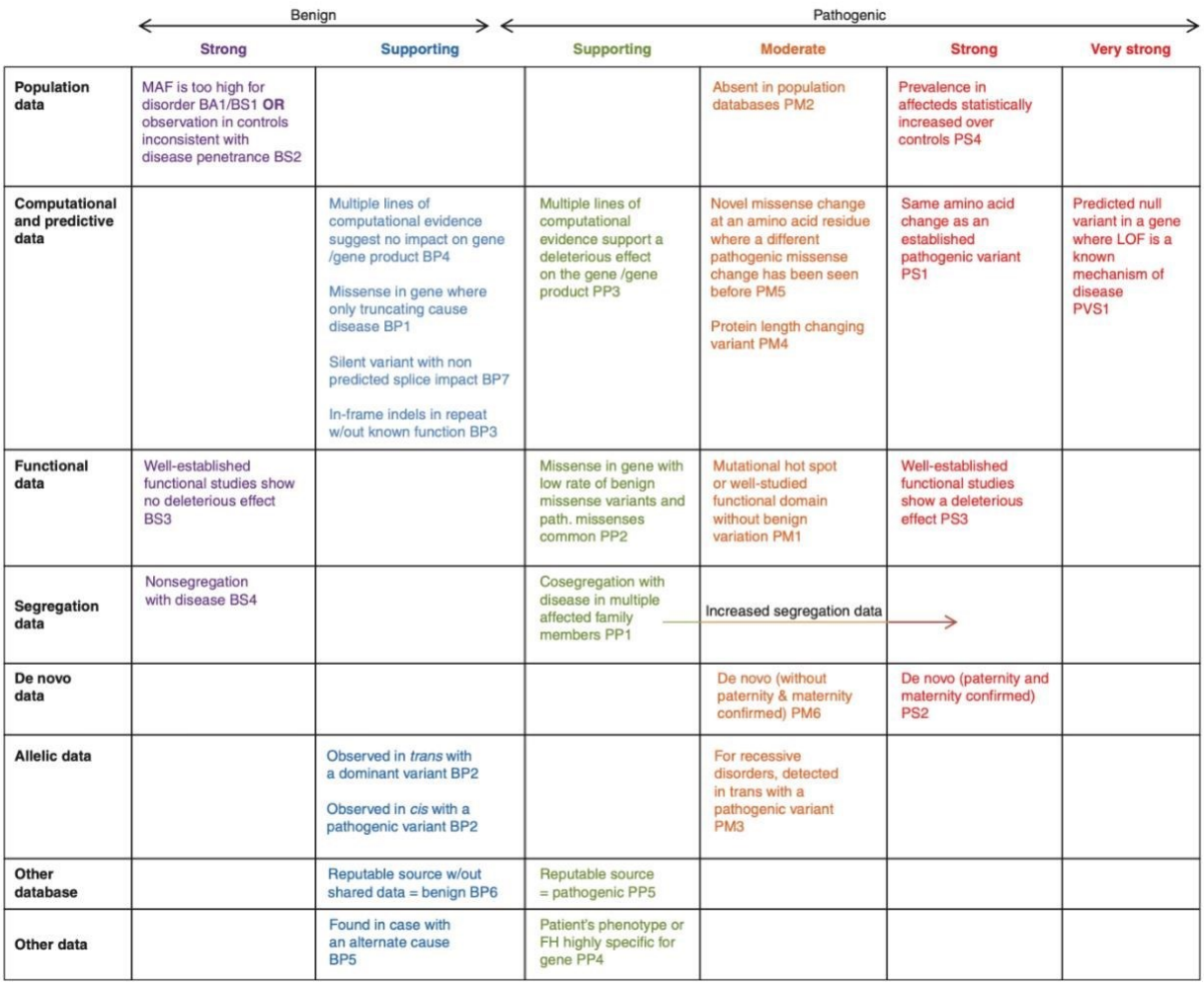


Figure 5. Evidence Framework for variant classification. BS, benign strong; BP, benign supporting; FH, family history; LOF, loss of function; MAF, minor allele frequency; path., pathogenic; PM, pathogenic moderate; PP, pathogenic supporting; PS, pathogenic strong; PVS, pathogenic very strong. Permissions to re-use figure obtained from the publisher. Retrieved from Richards et al., 2015.[51]

1.9. DNA methylation in SCCOHT

DNA methylation is the addition of a methyl group to cytosine residues in CpG dinucleotides. This mechanism plays a crucial role in regulating gene expression, and aberrant mechanism is a known hallmark of many cancers.[52] It is known that SWI/SNF complexes are involved in the

establishment of DNA methylation patterns.[53] Thus, DNA methylation profiles can serve as valuable biomarkers for cancer diagnosis, prognosis, and therapeutic targeting, due to the importance of the cellular environment in defining epigenetic characteristics. In SCCOHT, understanding the specific methylation changes associated with *SMARCA4* variants could shed light on the molecular mechanisms underlying the disease and distinguish it from other ovarian cancers. Previous studies have shown that SCCOHT demonstrates distinct methylation profiles from other ovarian cancers.[54] However, it has been reported that its methylation profiles show similarities with SMARCA4-deficient ATRT methylation profiles, suggesting similar mechanisms of action, likely linked to chromatin remodelling.[14] This finding further emphasizes the closely resemblance of these tumors. SMARCA4-deficient ATRTs are moreover molecularly distinct from SMARCB1-deficient cases.[55] These findings indicate that the cellular context is a critical determinant in shaping the epigenetic landscape, even in the presence of a core SWI/SNF deficiency.[54]Furthermore, comparing the methylation profiles of different *SMARCA4* variants, including truncating, missense, and splice variants, is essential to identify disease-specific methylation signatures for early detection and personalized treatment strategies.

1.10. Rationale and Research Objectives

1.10.1. Rationale

As discussed in section 1.7., it is not known why certain variants lead to a neurodevelopmental disorder, whereas others predispose to cancer. Correct classification of these variants is crucial for determining whether heterozygotes are at risk for cancer. However, significant issues exist today because there is no gene-specific classification for *SMARCA4.* Many variants are simply classed as Variants of Uncertain Significance (VUS), while others are classified as Likely Pathogenic/Pathogenic (LP/P) when they may not be. This is explained by the absence of

clinically relevant functional studies and a scarcity of clinical data due to the disease's rarity, resulting in an inability to make confident classifications for these variants' pathogenicity, resulting in problems and concerns with classification, particularly the classification of missense variations. This leads to important challenges in clinical management when the main risk-reducing intervention for unaffected females carrying these variants is preventive oophorectomy, which has a dramatic impact on quality of life. It is important that we do not recommend removal of the ovaries of a young woman who is not truly at risk.

1.10.2. Research objectives and aims

In response to this critical need for refinement, the overall aim of this thesis is to improve classification of SMARCA4 variants targeting predictive and functional assessment strategies.

Aim 1: Modification of ACMG/AMP Criteria and Reclassification of *SMARCA4* variants.

Aim 2: Molecular Analysis of Splicing Variants.

Aim 3: Using Functional Assays to determine the impact of *SMARCA4* variants.

Aim 4: Exploring DNA methylation profiling as a potential biomarker for *SMARCA4* variants.

1.10.3. Hypothesis and Research Plan

We hypothesize that through the integration of variant data, genomic data, expression data, and functional data, we can improve variant classification and patient management. We aim to create *SMARCA4*-specific classification criteria and reclassify as many variants as possible.

As the main plan for this thesis, we intend to collect variant data from multiple sources, integrating patient phenotypic profiles, leveraging a database of 2870 unique *SMARCA4* variants with the objective to create variant classification criteria that are specific for *SMARCA4* and RTPS2 with the aim of reclassifying as many *SMARCA4* VUSs as possible and

confirming that current LP/P classifications are correct. It should be noted that we have not attempted to classify *SMARCA4* variants with respect to pathogenicity for CSS. Through systematic analysis and frequency calculations, we present suggested modifications to certain classification criteria and a list of reclassified variants (Section 3.1). The reclassification will be supported by functional analysis of selected splicing variants where we had access to patient lymphocytes for analysis (Section 3.2).

In secondary aims of this thesis, we aim to use molecular assays (Section 3.3: Using Functional Assays to determine the impact of *SMARCA4* variants. Section 3.4: Exploring DNA methylation profiling as a potential biomarker for *SMARCA4* variants.) to investigate in greater depth the activity of selected variants with known pathogenicity. The long-term purpose of this approach is twofold: to better understand the molecular events caused by each variant to help with designing targeted therapies, and to determine whether it may be possible to establish rapid assays or identify biomarkers to assist with variant classification of variants of unknown significance clinically.

2. Materials and Methods

2.1. Ethics Declaration

The work performed in this thesis was approved by the Review Ethics Boards of the McGill University Health Centre and CIUSSS Centre-Ouest (MP-37-2019-5465, Genome-Wide Approaches in Hereditary Cancer Families; 2021-6537, SCCOHT/SMARCA4 Registry and Biobank; and MP-05-2016-404, Towards a biological understanding of small cell carcinoma of the ovary, hypercalcemic type).

2.2. Collection of Patient Data and Samples

The present study collected variant information (VUS/LP/P) identified in *SMARCA4*, along with basic patient phenotype data, from nine sources. We obtained variant data (n total = 2870) from the following clinical testing labs: Ambry Genetics (n = 1150), Baylor Clinical Laboratory (n = 356), The University of Chicago Genetic Services Laboratories (n = 26), Geisinger Health Services (n = 22), GeneDx (n = 52), Invitae (n = 1258), and Prevention Genetics (n = 6). We also obtained data from 10 published studies[9,15,18,26,44,48,56-59] and samples and data from our local SCCOHT-SMARCA4 Registry and Biobank directed by Dr Foulkes at the RIMUHC.[60]

2.3. Variant annotation and modification of ACMG/AMP criteria

Once all aforementioned variants were compiled, the variants were annotated on *SMARCA4* NM_001128849.3 transcript. Variant classifications from ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), a freely accessible, public archive of reports of human variations classified for diseases and drug responses, along with a comprehensive literature review, were incorporated into the analysis. We made specifications to the

ACMG/AMP criteria for *SMARCA4* to better allow for definitive classification of variants. We based these modifications on those from other ClinGen Variant Curation Expert Panels (VCEPs; PTEN,[61] TP53,[62] CDH1,[63] DICER1[64]) and our collected datasets. Expert panels in variant curation assess the evidence to classify a genomic variant on a scale from pathogenic to benign, considering a specific disease and its inheritance pattern.

2.3.1. Assessment of biologically irrelevant exons

In collecting variants from thousands of individuals, we noticed some trends of discrepancies between the phenotypes and the pathogenicity predictions for variants in certain regions of the gene, notably in exons 27 and 30 on transcript NM_001128849.3. To study the biological relevance of these exons in the context of our *SMARCA4* variant classification rules, *in silico* analysis was performed using GTex[65] and UCSC databases.[66] RNA-seq data from blood and tissue from unaffected healthy individuals, from ECRT and ATRT samples, and from the SCCOHT-1 and BIN67 cell lines was visualized using IGV.[67] Additionally, cDNA was generated from RNA samples from blood and ovarian tissues obtained from unaffected patients following the manufacturer's protocol (ThermoFisher Scientific - SuperScript™ IV Reverse Transcriptase). Sanger sequencing was done on targeted regions of the cDNA (see section 2.4, **Table 1**).

2.3.2. Testing of splicing variants

To elucidate the role and pathogenicity of specific splicing variants in exon 28 of *SMARCA4* transcript NM_001128849.3, we analyzed amplified regions for c.3951+2T>C and c.3951+1G>A variants from cDNA (See **Table 1)**. The lab obtained lymphoblastoid cell lines (LCLs) from three individuals for study: a proband with the c.3951+2T>C variant, a negative (non-carrier) aunt as control, and the SCCOHT patient with the c.3951+1G>A variant. The

experiments were started by other lab members, including treatment of growing cells with cycloheximide which inhibits nonsense-mediated decay of transcripts bearing truncating mutations (NMD). RNA was extracted from these LCLs by a previous lab member. I synthesized cDNA from these RNA samples following the manufacturer's protocol (ThermoFisher Scientific - SuperScript™ IV Reverse Transcriptase) and performed targeted PCR amplification. Sanger sequencing was performed on the PCR amplicon (see section 2.4, **Table 1**).

2.4. PCR and Sanger sequencing

Sanger sequencing was used to validate variants. Primer pairs for PCR amplification and sequencing were designed using Primer3 (https://primer3.ut.ee/) and were purchased from IDT (https://www.idtdna.com) (**Table 1**). All PCR reactions were done using Qiagen HotStarTaq Plus DNA Polymerase (#203605), 10mM dNTP, and 10x PCR Buffer reagents with 5 µM primers in a 25 µL reaction with the following parameters: Step 1: 6 min 95°C, Step 2: 1 min 95°C, Step 3: 1 min 59°C, Step 4: 1 min 72°C, repeat Step 2 to Step 4 31 times, Step 5: 10 min 72° and Step 6: keep at 10°C. PCR products were visualized by gel electrophoresis in a 1.5% agarose gel with GelRed (Biotium). Products were purified using Qiagen QIAquick Gel Extraction Kit and sequenced by conventional Sanger methods as a service by the Centre d'expertise et de services Génome Québec in Montreal. Sequences were analyzed using the Unipro UGENE software (Unipro UGENE).

Table 1. Primer list

Primer Name	Primer Sequence	Section
26F	5' ACAGCGTGGAGGAGAAGATC 3'	Sections 3.2. and 3.3.

		(Methods 2.3.1., 2.3.2. and 2.4.)
29Rv2	5' TCAGTGAGTCGCTGTAGTCCAC3'	Sections 3.2. and 3.3. (Methods 2.3.1., 2.3.2. and 2.4.)

2.5. Functional Assays

We conducted functional assays to validate and categorize some variants. In these assays, we use selected benign, CSS-related, ATRT-related and SCCOHT-related variants as a reference. We also use an existing SCCOHT cell line (SCCOHT-1)[32] defective in SMARCA4 to express benign, non-truncating, and CSS-related *SMARCA4* variants with known (reference) or unknown (variants under investigation) pathogenicity. The SCCOHT-1 cells were infected with lentivirus containing empty vector, *SMARCA4* VUS or controls. To enable cross-comparison with controls, the same infection multiplicity was employed.

To determine the impact of these variants on the behaviour of the SCCOHT cell line, established functional assays were used, including DNA methylation assays, protein expression assays, and cell viability assays. Some assays were started during my project but are still ongoing in the lab. The cell viability and protein expression assays are ongoing projects in our lab and that of collaborator Dr Sidong Huang (Section 3.3). For the DNA methylation assay, important controls were still missing at the time of writing this thesis (see Section 3.4 for details).

2.5.1. Cell culture

The SCCOHT-1 and HEK293T cell lines were obtained from Dr. Sidong Huang (McGill University, Montreal). The SCCOHT-1 cell line was cultured in RPMI with 7% Fetal Bovine Serum (FBS), penicillin/streptomycin and 2mM L-glutamine. The HEK293T cell line was cultured in DMEM with 7% FBS, penicillin/streptomycin and 2mM L-glutamine. The cell lines

were free of Mycoplasma and were maintained 37 degrees Celsius and 5% CO_2. Cells passage took place every 2-3 days.

2.5.2. Viral Transduction

Lentiviral transduction was performed using the protocol as described at http://www.broadinstitute.org/rnai/public/resources/protocols. Infected cells (30 hours post-infection) were selected in puromycin for 1-2 days (when all cells in the selection control died) and harvested immediately for the experiments. SCCOHT-1, a SCCOHT cell line, defective in SMARCA4, was used to express benign, missense, and CSS-related *SMARCA4* variants. SCCOHT-1 cell line was infected with lentivirus containing empty vector, *SMARCA4* VUS or controls. A total of 11 variants were studied (**Table 2**). S1, S2 and S3 represent non-truncating variants seen in SCCOHT patients. CSS1 and CSS2 represent non-truncating variants seen in CSS patients. ATRT1 and ATRT2 represent non-truncating variants seen in ATRT patients. The ATPase dead variant represents a variant that leads to decreased ATPase activity. We included this variant to test the hypothesis that a damaged ATPase may play a role in SCCOHT oncogenesis. Benign and Wild type (WT) are used as positive controls. The empty vector (Lv) is used as control. The plasmids were provided by Dr. Sidong Huang (McGill University, Montreal), who purchased them from Bio Basic. Plasmids were received in the form of bacterial cultures and were maxiprep following manufacturer's protocol (QIAGEN Plasmid Maxi Kit).

Table 2. 11 Plasmids used for lentiviral transfection.

Plasmid name	Information	Amino Acid change
S1	pReceiver_SMARCA4_c.2311-2316del	p.Asn771_Asn772del

S2	pReceiver_SMARCA4_c.3610_3615del	p.Leu1204_Cys1205del
S3	pReceiver_SMARCA4_c.3239G>A	p.Gly1080Asp
CSS1	pReceiver_SMARCA4_c.3380A>G	p.Asp1127Gly
CSS2	pReceiver_SMARCA4_c.1636-1638del	p.Lys546del
ATRT1	pReceiver_SMARCA4_c.2335G>A	p.Asp779Asn
ATRT2	pReceiver_SMARCA4_c.2491_2505del	p.Lys831_Lys835del
ATPase dead	pReceiver_SMARCA4_c.2354A>G	p.Lys785Arg
Benign	pReceiver_SMARCA4_c.1114T>C	p.Tyr372His
WT	pReceiver_SMARCA4	NA
Lv	Empty vector as control	NA

NA, Not Applicable; S, Small-cell carcinoma of the ovary, hypercalcemic type; CSS, Coffin-Siris Syndrome; ATRT, atypical/typical rhabdoid tumor; WT, Wild-Type.

2.5.3. Cell viability and Protein expression assays

Cells from the paragraph 2.5.2 were trypsinized and replated for protein expression assays, short-term cell viability assays, and long-term cell viability assays.

Compounds and antibodies:

The following antibodies were used: Beta-Actin and HSP90 as control; Cyclin D1, a regulatory subunit of cyclin-dependent kinases CDK4 and CDK6[32]; BRM (SMARCA2) and BRG1 (SMARCA4), ATPase subunits of SWI/SNF; CD44, a cell surface adhesion receptor that is highly expressed in many cancers and regulates metastasis via recruitment of CD44 to the cell surface [68]; IP3R3, a protein encoded by the ITPR3 gene, releasing calcium ion (Ca2+) from the endoplasmic reticulum (ER) into the cytosol.[33] Antibodies were purchased from Santa Cruz (HSP90, Beta-actin and Cyclin D1), AbCam (BRG1), BD Biosciences (IP3R3) and Cell

Signaling (BRM); Antibody against SMARCA4 was used with 1:5000 dilution and all others with 1:1000 dilution. The cells from paragraph 2.5.2 were washed with 1% PBS and trypsinized with 0.05% trypsin. Following this, the cells were counted. For each of the various assays, the appropriate quantity of cells was plated.

Protein expression assay:

Cells were seeded into 6-well plates (500k per well) or 12-well plates (200k per well), depending on proliferation rate and cell size, and were cultured. The next day, cells were washed with cold PBS, lysed with protein sample buffer and processed with Novex® NuPAGE® Gel Electrophoresis Systems (Invitrogen). HSP90 and Beta-actin served as loading controls.

Short-term Cell viability assay:

Cultured cells were seeded into 96-well plates (4k per well). Cells were then incubated for 5-7 days, and cell viability was measured using the CellTiter-Blue viability assay (Promega). 20µl/well of CellTiter-Blue® Reagent was added. Plates were incubated using standard cell culture conditions for 1–4 hours. Fluorescence was recorded at 560/590nm.

Long-term Cell viability assay:

Single cell suspensions of SCCOHT-1 cells were seeded into 6-well plates (40k per well) and were cultured. At the endpoints of colony formation assays (14 days), cells were fixed with 3.75% formaldehyde, stained with crystal violet (0.1%w/v) and photographed.

2.5.4. DNA Methylation Profiling

Cells from the previous paragraph were trypsinized and gDNA were extracted by following the manufacturer's protocol (QIAGEN Blood & Cell Culture DNA Mini Kit). gDNA of the variants in the SCCOHT-1 cell line were concentrated at 100ng/ul with a minimum of 35ul. gDNA was also collected from patients' blood. DNA methylation assays will be used to identify differentially methylated genes (DMGs) in the cell line and in lymphocytes from patients (via our SCCOHT/SMARCA4 Registry (sccoht-smarca4.ca)) carrying these variants to enable correlation of DMGs. The Centre d'expertise et de services Génome Québec will perform sodium bisulfite conversion and will then process and analyse the samples on the Infinium HumanMethylationEPIC BeadChip (Illumina 850K). The resulting data will then be converted into b-values, determined from the ratio of the methylated signals vs. the total sum of unmethylated and methylated signals, ranging between 0 and 1, using the minfi Bioconductor package in R.

3. Research Findings

3.1. Modification of ACMG/AMP Criteria and Reclassification of *SMARCA4* variants

The objective of this aim was to leverage our research team's unique expertise with *SMARCA4* to update how ACMG/AMP criteria are applied to variants in *SMARCA4* in the context of SCCOHT. The findings are being submitted as a manuscript.

3.1.1. Criteria used to assess pathogenicity and benignity

We modified certain ACMG/AMP codes to make them more specific to *SMARCA4* and RTPS2. **Table 3** found at the end of this section summarizes the ACMG/AMP code modifications we made. Codes that remained unmodified are PS1, PS2, PM6, PP1, PP3, BS4, BP2, BP4, BP5 and BP7. Codes that are not applicable for the gene include PM1, PM3, PP2, PP4, PP5, BS3 and BP6. For the remaining codes, the modifications that were made are detailed in the following paragraphs.

3.1.1.1 PVS1 - Not applicable to LoF variants in exons 27 and 30 in NM_001128849.3, and RNA evidence for splicing variants

For PVS1, we followed the recommendations from the Sequence Variant Interpretation (SVI) Working Group[69] and from the ClinGen SVI Splicing Subgroup[70] with certain modifications. Firstly, PVS1 cannot be applied to LoF variants in exons 27 and 30 on transcript NM_001128849.3, as these exons are not present in all transcripts and are not biologically relevant (**Figure 6**). Sanger sequencing of cDNA from blood and ovarian tissue samples in unaffected individuals shows that exons 27 and 30 are not expressed in these tissues (**Figure 7**). RNA sequencing data from SCCOHT cell lines further demonstrate that these exons are not

incorporated into transcripts expressed in these tumors (**Figure 8**). Moreover, in silico data from GTex confirm the low expression of these two exons in normal ovarian tissue.[65] RNA sequencing data from *SMARCA4*-mutated extra-cranial rhabdoid tumors (ECRT) and ATRT also show low expression of exons 27 and 30 (**Figure 9**). This modification applies for variants causing SCCOHT, ATRTs and MRTs.

In addition, PVS1 cannot be applied to LoF variants downstream of codon 1593, as the last codon at which a pathogenic LoF variant has been seen in an RTPS2-related tumor is a frameshift at p.Val1593 (NM_001128844.3: c.4667del), with a premature stop codon predicted 35 amino acids downstream, reported in a 2-year-old female with ATRT.[71]

Moreover, PVS1_Strength (RNA) criterion was applied for splicing variants, as recommended by Walker et al.[70] For purposes of application of the PVS1 code for splicing variants, the ATPase domain (exons 16-25) is considered a critical domain.

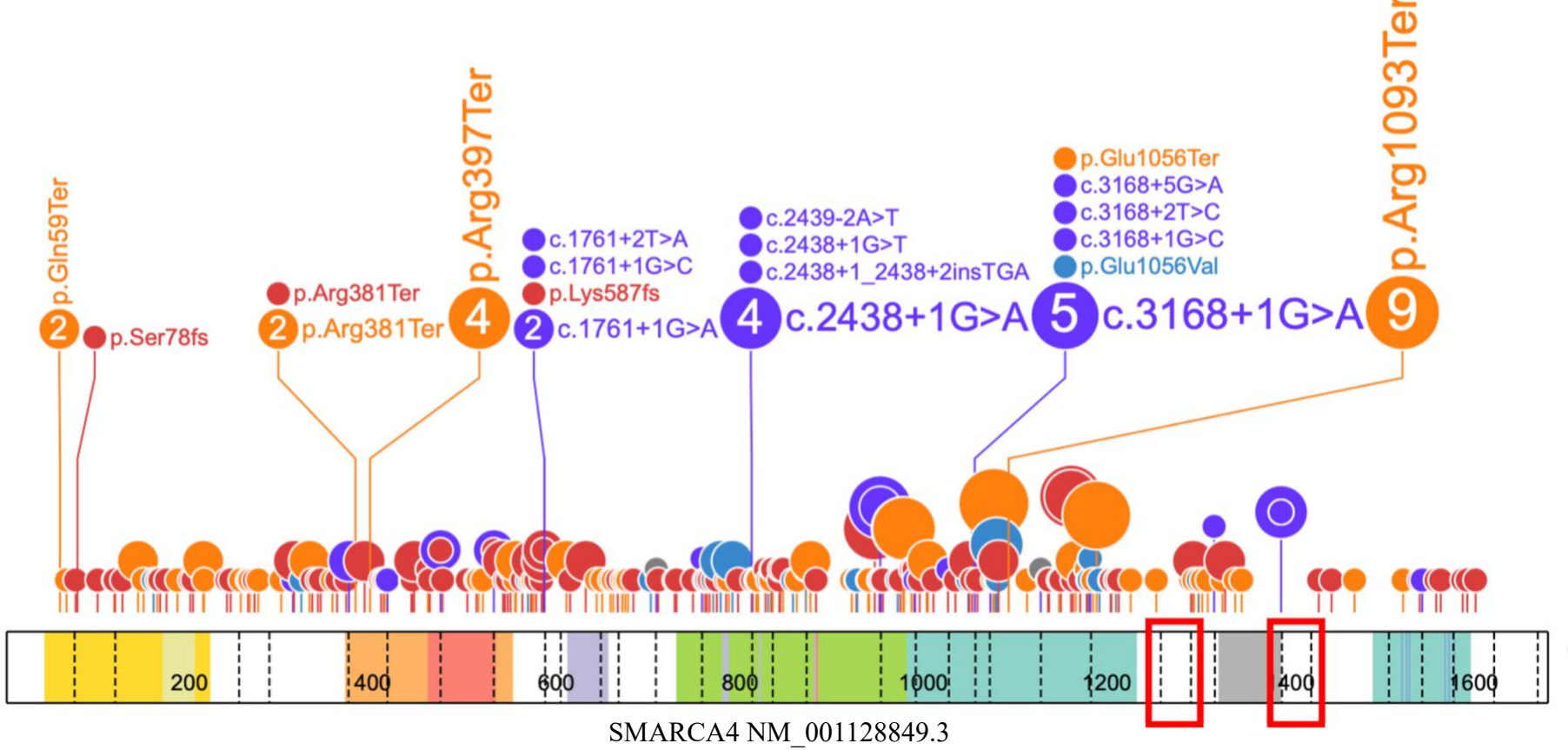


Figure 6. Location of variants found in SMARCA4 in SCCOHT, ATRTs and MRTs. Lolliplot of SMARCA4 protein (transcript NM_001128849.3) with variants identified in SMARCA4 in SCCOHT, ATRT and MRT. Numbers in each lollipop correspond to the number of times the variant was seen. Orange = nonsense variants, Red = frameshift variants, Purple = splice variants, Blue = missense variants and Grey = protein indel variants. Exons 27 and 30 are surrounded by red boxes, showing no variants in these regions.

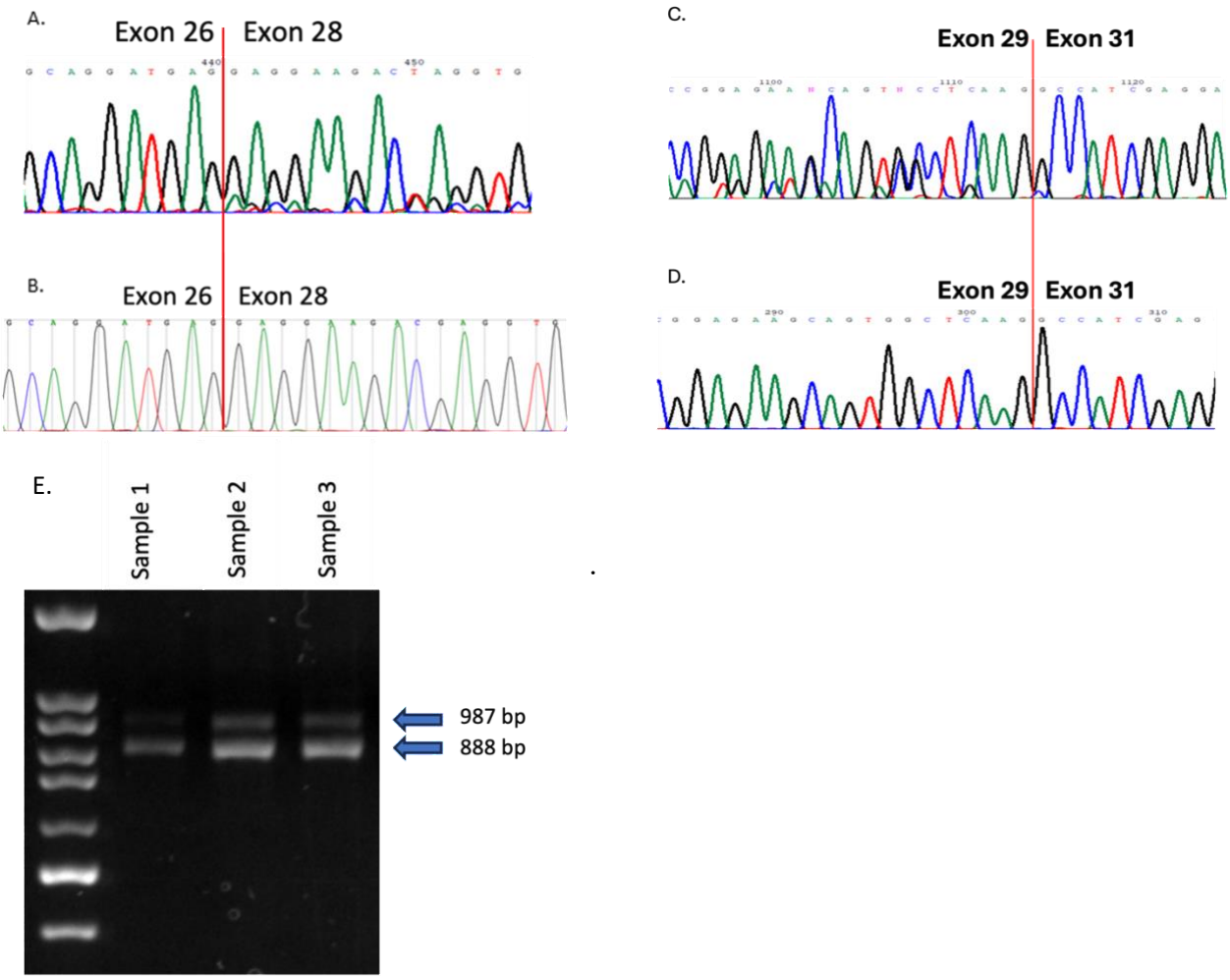


Figure 7. cDNA analysis of exons 27 and 30. A) Sanger sequencing of cDNA from blood in a healthy individual shows very low expression of exon 27. B) Sanger sequencing and agarose gel of cDNA from normal ovary shows no expression of exon 27. C) Sanger sequencing of cDNA from blood in a healthy individual shows very low expression of exon 30. D) Sanger sequencing of cDNA from normal ovary shows no expression of exon 30. E) Agarose gel of cDNA amplification across exon 27 from three normal ovarian samples shows that exon 27 is expressed at a low level. The bands represent transcripts with exon 27 (987 bp) and without exon 27 (888 bp) incorporated. Note that Sanger traces shown in (B) are from sequencing of the 888 bp band only, with no exon 27 incorporated. Sequencing of the weakly expressed 987 bp band showed incorporation of exon 27 (not shown).

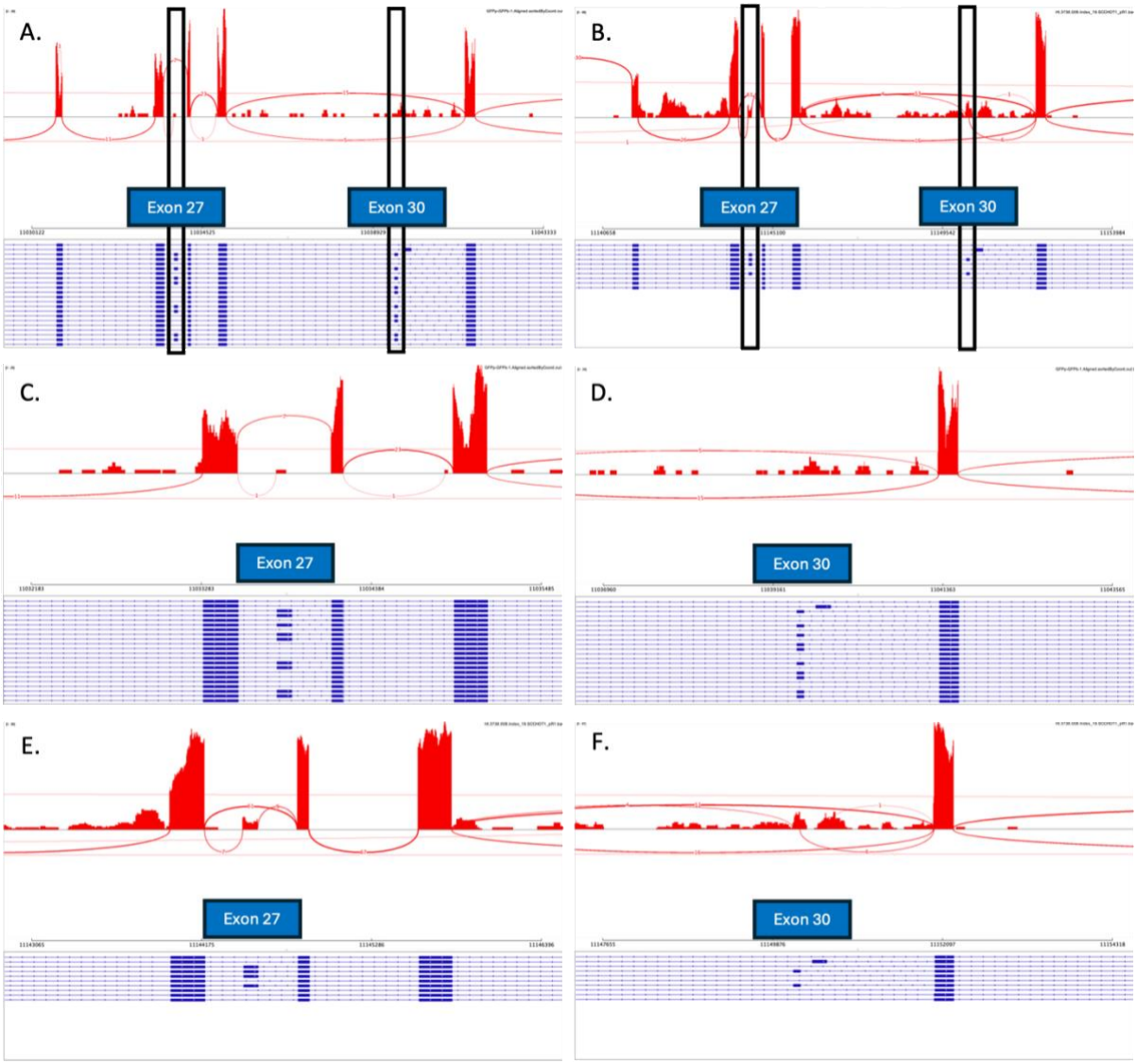


Figure 8. RNA sequencing data in BIN-67 and SCCOHT-1 cells lines. Sashimi plots from RNA sequencing data in SCCOHT cell lines (BIN-67 (A) and SCCOHT-1 (B)) show low expression of exons 27 and exon 30 (transcript NM_001128849.3). Black boxes surround exons 27 and 30. (C+D) Enlarged regions of Exons 27 (C) and 30 (D) in BIN-67 cell line. (E+F) Enlarged regions of Exons 27 (E) and 30 (F) in SCCOHT-1 cell line.

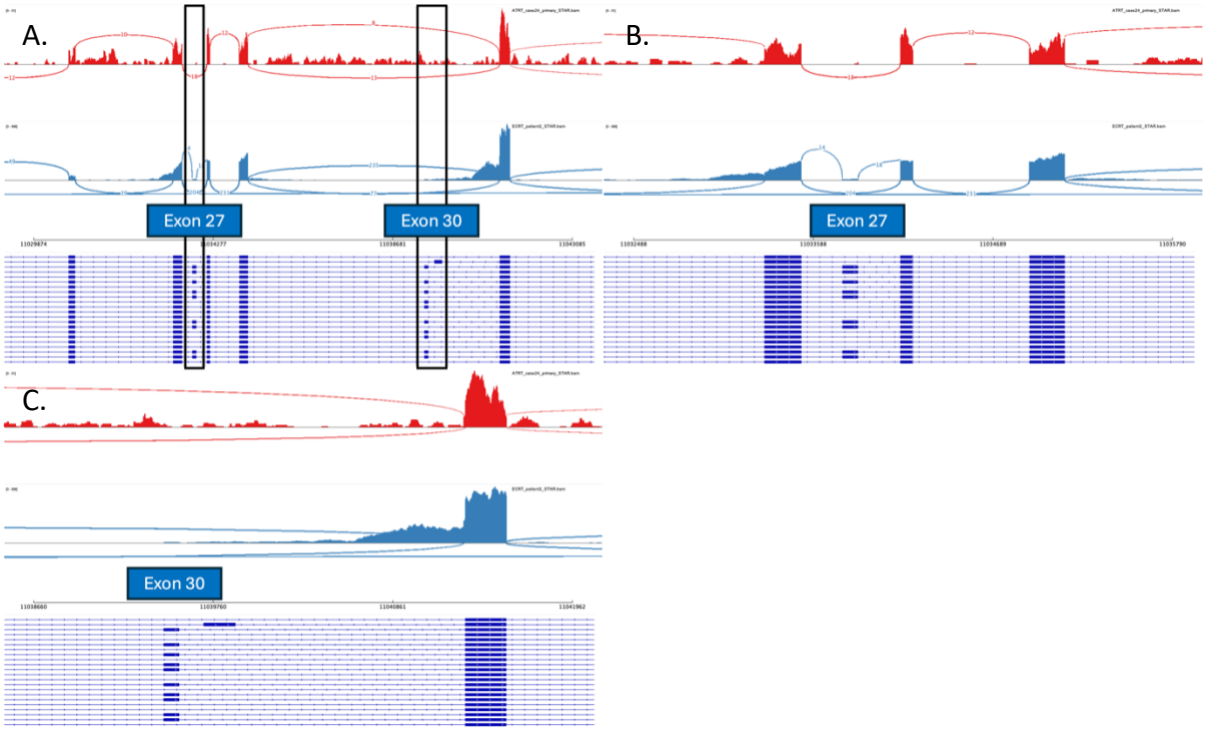


Figure 9. RNA sequencing data in ATRT and ECRT samples. A) RNA sequencing data of ATRT (top) and SMARCA4-mutated extra-cranial rhabdoid tumors (ECRT) (bottom) shows low expression of exons 27 and 30 (transcript NM_001128849.3). Black boxes surround exons 27 and 30. (B+C) Enlarged regions of Exons 27 (B) and 30 (C).

3.1.1.2.PS3 – Functional evidence and RNA analysis for missense variants

PS3 is only applicable for missense variants, as recommended by the ClinGen SVI Splicing Subgroup.[70] As there are no established biologically relevant assays for *SMARCA4*, we followed the recommendations by Brnich et al for PS3.[72]

3.1.1.3.PS4 – Observation of the variant in multiple affected individuals and variants seen in SCCOHT/ATRT/MRT tumors

The original PS4 code was designated for variants where the prevalence of the variant in affected individuals is significantly increased compared with the prevalence in controls. However, with rare diseases, the ability to perform odds ratios is impractical, and therefore

many VCEPs for genes associated with rare disease have instead opted for a proband counting strategy.[61,62,63,64] Similarly, we implemented a tumor and proband counting technique with a point system for *SMARCA4* and its associated phenotypes. We assigned one point if the variant is seen in one proband with pathologically confirmed SCCOHT, MRT, or ATRT. Each additional proband with the variant observed contributes an incremental point. This was applied for variants in the germline of a proband but was not applied if the variant met criteria for BS1/BA1, as previously recommended by Mester et al.[61]

Moreover, as *SMARCA4* is pathognomonic for SCCOHT, and one of only two genes known to cause ATRT and MRT (along with *SMARCB1*),[73] we also applied the PS4 code for variants seen in one of these three tumor types. One point is assigned if a variant is observed in a pathologically confirmed RTPS2-related tumor where SMARCA4 protein expression is retained (no second hit) or if tumor sequencing was not performed. Although retention of SMARCA4 expression might indicate that the variant is not causing disease, due to the monogenic nature of these tumors, and the fact that some pathogenic missense or splice variants do result in retention of expression,[19] we felt that it was appropriate to incorporate this piece of evidence due to the specificity of the gene-disease association. If a variant is seen in a RTPS2-related tumor with loss of SMARCA4 expression in the tumor and in the absence of two other tumor-confined, P/LP variants (second hits), one extra point is obtained. Similarly, if a variant is seen in a RTPS2-related tumor and there is loss of heterozygosity (LOH) of the wild-type allele, one extra point is obtained. Points are cumulative across tumors and probands. Addition of all points allows for classification: supporting evidence (PS4_P) equals to one point, moderate evidence (PS4_M) equals to one point 2-3 points and strong evidence (PS4_S) equals to 4+ points

It is important to note that this should be used when the variant is detected on tumor testing, even if the origin of the variant is confirmed somatic or unknown, as the mechanism

of disease is the same for both germline and somatic variants, and these tumors are somatically quiescent.[74]

3.1.1.4.PM5 – Missense variant at same codon as another pathogenic missense variant

We applied PM5 at a moderate level (PM5_M) if a missense variant is present at the same amino acid as another pathogenic missense variant, or at a strong level if ≥2 known pathogenic missense variants were present at the same position. Notably, these variants must be pathogenic for RTPS2-related tumors using these rules, not for CSS. Similar modifications have been applied to other genes where disease mechanisms and variant impacts are well-understood, such as TP53. For instance, both c.818G>A (p.Arg273His) and c.818G>C (p.Arg273Pro) are pathogenic variants at codon 273. In *SMARCA4*, both c.4170G>C (p.Lys1390Asn), seen in a patient with MRT, and c.4170G>A (p.Lys1390Lys), seen in a patient with ATRT, are likely pathogenic variants at codon 1390.

3.1.1.5.BA1/BS1 – Allele frequency thresholds for SMARCA4 in SCCOHT

To calculate cut-offs for BA1 and BS1 for *SMARCA4*, we used the formula (prevalence x heterogeneity)/penetrance specified by Whiffin et al.[75] Although the exact prevalence of SCCOHT is unknown, it constitutes less than 0.01% of all ovarian malignancies.[6] Therefore, the prevalence used was 1/1,000,000. The penetrance of *SMARCA4* variants in SCCOHT has not been established, however it is estimated to be high, given the paucity of pathogenic variants present in gnomAD. To be conservative, we estimated a penetrance to age 50 of 20%.[76] The genetic heterogeneity was set to 0.5 to take into account ATRT and MRT, as they are more commonly caused by *SMARCB1* variants. For the allelic heterogeneity, the most frequent variant we have seen to cause an RTPS2-related tumor was found in 8 of 272 cases (0.03), and therefore to be conservative we set the allelic heterogeneity to 0.3. Using these numbers, we

calculated a credible maxAF of $3.75x10^{-7}$ and therefore used a maxAF of $3.75x10^{-6}$ to be conservative. Using the highest allele count in gnomAD v4.1 for *SMARCA4* (AC=1614104), this gave a maximum credible allele count of 10 individuals. Therefore, for BA1, a maxAF >3.75e-6 with an AC >10 females was applied. For BS1, we used the less conservative estimates of 0.1 for allelic heterogeneity and 30% for penetrance. This gave us a maxAF of 1.2e-7and AC >5 females.

3.1.1.6.BS2 – Variant observed in unaffected females or in homozygotes

Assuming a penetrance to age 50 of 20%, we used BS2 at a supporting level if a variant was seen in 5 or more unaffected females over the age of 50, or if it was seen in one or more homozygotes in gnomAD, as homozygous pathogenic *SMARCA4* variants are thought to be embryonic lethal. BS2 was applied at a moderate level if the variant appeared homozygous in two individuals but homozygosity status was not confirmed. It was elevated to a strong level if homozygous status was confirmed in one individual, or if the variant was seen in 10 or more unaffected females over the age of 50, and at very strong level if homozygous status was confirmed in two or more individuals.

3.1.1.7.BP1 – Exception of ATPase domain for missense variants

We applied BP1 at a supporting level as per ACMG/SVI guidelines, unless the missense variant is located in the ATPase domain (from residue 766 to 1246), as there are no known pathogenic missense variants located outside this region.

3.1.1.8.BP3 – In-frame indels

This code can be applied at moderate strength to in-frame insertions and deletions from p.Gly229 to p.Pro244. This region of the gene contains 12 Gly-Pro repeats and many in-frame

insertions and deletions have been seen in this region in unaffected individuals. Given the number of variants in this region of SMARCA4 in gnomAD, it would not be expected to disrupt the protein's structure in a manner that would result in pathogenicity, supporting the use of BP3 at a moderate strength (BP3_M) for these specific in-frame changes. Similar region-specific criteria have been applied to other genes with repetitive regions where variations are often benign, such as the *VHL* gene (applied to the 8x GXEEX AA repeat motif in the 5' end of VHL p30) and the *FOXG1* gene (applied to poly His, poly Gln, and poly Pro regions, which are repetitive regions that are known to be variable in the normal population).

3.1.2. Reclassification of non-truncating variants

3.1.2.1. Likely pathogenic variants to VUS

In testing exons 27 and 30 we were able to determine that variants in these exons were unlikely to predispose to SCCOHT, MRT and ATRT, as they are not expressed in the ovary (**Figures 7, 8, 9**). Loss of function variants in these exons should currently be classified as VUS, as their clinical significance is unknown. See **Table 4** for details on these variants.

3.1.2.2. VUS/Conflicting to Likely Benign or Benign

Using SMARCA4-specific ACMG/AMP rules, we were able to downgrade 40 non-truncating variants that were classified in ClinVar as VUS or had conflicting classifications to Likely Benign or Benign variants, mostly due to their frequency in gnomAD v4.1 or in unaffected individuals in our dataset. Additionally, we have determined that in-frame insertions and deletions in exon 4 of SMARCA4 within a repetitive region (p.Gly229 to p.Pro244) should be classified as likely benign based on their incidence in 441 unaffected individuals. See **Table 4** for details on these variants.

3.1.2.3. VUS/Absent from Clinvar to Likely Pathogenic

Using these rule specifications, we classified eight missense variants as Pathogenic or Likely Pathogenic. These variants were previously classified as either VUS (n = 3), absent from ClinVar (n = 4), or known to be Pathogenic. These reclassifications were primarily based on their occurrence in RTPS2 probands and supported by computational evidence indicating their deleterious effect on the gene or gene product.

3.1.2.4. Absent from Clinvar to VUS

Using these rule specifications, we classified six missense variants seen in patients with SCCOHT, ARTR and MRT, that were previously absent from ClinVar as VUS.

3.1.3. Classification of truncating variants

Through the acquisition of variants from various sources, we also used these rules to classify 64 truncating variants seen in 73 previously unreported patients with SCCOHT, MRT, or ATRT (**Supplementary Table S1**).

Table 3. *SMARCA4*-modified ACMG/AMP specification.

PATHOGENIC CRITERIA						
Criteria	**Original**	**Modifications**				
		Stand-alone	**Very strong**	**Strong**	**Moderate**	**Supporting**
PVS1	Null variant in a gene where LOF is a known mechanism of disease	Full gene deletion	As per ACMG/SVI guidelines. Cannot be applied to LoF variants in exons 27 and 30 in transcript NM_001128849.3, as these exons are not present in all transcripts			
PVS1_Strength (RNA)	-	-	As per recommendations from Walker et al. ATPase domain (exons 16-25) is considered a critical domain.			
PS1	Same amino acid change as a previously established pathogenic variant regardless of nucleotide change	As per ACMG/SVI guidelines.				
PS2	De novo (both maternity and paternity confirmed) in a patient with the disease and no family history	As per ACMG/SVI guidelines.				
PS3	Well-established in vitro or in vivo functional studies supportive of a damaging effect on the gene or gene product	Not applicable for splicing variants				In vitro study showing that the variant leads to absent SMARCA4 expression. Only applicable to missense variants.
PS4	Prevalence of variant in affected individuals > prevalence in controls	-	-	4+ points	2-3 points	1 point
PM1	Missense mutation located in a mutational hot spot and/or critical and well-established functional domain without benign variation	Not Applicable				
PM2	Absent from controls in Exome Sequencing Project, 1000 Genomes Project, or Exome Aggregation Consortium	Absent from the most recent version of gnomAD				
PM3	For recessive disorders, detected in trans with a pathogenic variant	Not Applicable				
PM4	Protein length changes as a result of in-frame deletions/insertions in a nonrepeat region or stop-loss variants	As per ACMG/SVI guidelines. Not applicable to in-frame indels between p.Gly229 and p.Pro244				
PM5	Novel missense change at an amino acid residue where a different	-	-	Missense variant at a	Missense variant at same codon as pathogenic	-

	missense change determined to be pathogenic has been seen before			codon with ≥2 pathogenic missense variants predicting different amino acid change.	variant predicting a different amino acid change.	
PM6	Assumed de novo, but without confirmation of paternity and maternity	As per ACMG/SVI guidelines.				
PP1	Cosegregation with disease in multiple affected family members in a gene definitively known to cause the disease	As per ACMG/SVI guidelines.				
PP2	Missense variant in a gene that has a low rate of benign missense variation and in which missense variants are a common mechanism of disease	Not Applicable				
PP3	Multiple lines of computational evidence support a deleterious effect on the gene or gene product	As per ACMG/SVI guidelines.				
PP4	Patient's phenotype or family history is highly specific for gene	Not Applicable				
PP5	Reputable source recently reports variant as pathogenic, but the evidence is not available to the laboratory to perform an independent evaluation	Not Applicable				
BENIGN CRITERIA						
Criteria	**Original**	**Modifications**				
		Stand-alone	**Very strong**	**Strong**	**Moderate**	**Supporting**
BA1/BS1	Allele frequency >5%/Allele frequency > expected for disorder	Max AF>3.75e-6 AC>10	Max AF >1.2e-7 AC>5	-	-	-
BS2	Observed in a healthy adult individual for a recessive (homozygous), dominant (heterozygous), or X-linked (homozygous) disorder, with full penetrance expected at early age	-	Homozygous status confirmed in ≥2 individuals	variant seen in 10+ unaffected females over the age of 50, or homozygous status	homozygous in 2 individuals but status not confirmed	variant seen in 5+ unaffected females over the age of 50, or seen in ≥1 homozygotes in gnomAD

				confirmed in 1 individual		
BS3	Well-established in vitro or in vivo functional studies show no damaging effect on protein function or splicing	Not applicable				
BS4	Lack of segregation in affected members of a family	S As per ACMG/ VI guidelines.				
BP1	Missense variant in a gene for which primarily truncating variants are known to cause disease	-	-	-	-	Applicable for variants outside the ATPase domain (residue 766 to 1246)
BP2	Observed in trans with a pathogenic variant for a fully penetrant dominant gene/disorder or observed in cis with a pathogenic variant in any inheritance pattern	As per ACMG/SVI guidelines.				
BP3	In-frame deletions/insertions in a repetitive region without a known function	-	-	-	In-frame insertions and deletions from p.Gly229 to p.Pro244	-
BP4	Multiple lines of computational evidence suggest no impact on gene or gene product	As per ACMG/SVI guidelines.				
BP5	Variant found in a case with an alternate molecular basis for disease	As per ACMG/SVI guidelines. Applicable for SCCOHT/ATRT/MRT with pathogenic SMARCB1 variants detected, or other cancer where pathogenic variant explains disease.				
BP6	Reputable source recently reports variant as benign, but the evidence is not available to the laboratory to perform an independent evaluation	Not Applicable				
BP7	A synonymous (silent) variant for which splicing prediction algorithms predict no impact to the splice consensus sequence nor the creation of a new splice site AND the nucleotide is not highly conserved	As per ACMG/SVI guidelines.				

LoF, loss-of-function; AF, Allele Frequency; AC, Allele Count; SCCOHT, Small-cell carcinoma of the ovary, hypercalcemic type; ATRT, atypical/typical rhabdoid tumor; MRT, malignant rhabdoid tumor

Table 4. Variants re-classified using the proposed *SMARCA4*-specific ACMG/AMP modifications.

cDNA	Amino Acid	Exon	ClinVar classification	Our classification	Codes used	Age at diagnosis (yrs)	Tumor Type	PMID
Variants re-classified as Benign/Likely Benign								
c.76G>A	p.Ala26Thr	2	Conflicting	Benign	BA1, BS2, BP4_P	NA	NA	NA
c.79A>G	p.Met27Val	2	VUS	Likely Benign	BS1	NA	NA	NA
c.95C>T	p.Pro32Leu	2	VUS	Benign	BA1	NA	NA	NA
c.104C>T	p.Ser35Leu	2	Conflicting	Benign	BA1	NA	NA	NA
c.115G>A	p.Ala39Thr	2	Conflicting	Benign	BA1, BS2_P, BP4_P	NA	NA	NA
c.134C>A	p.Pro45His	2	VUS	Likely Benign	BS2_P	NA	NA	NA
c.207G>T	p.Met69Ile	2	VUS	Likely Benign	BS1	NA	NA	NA
c.235A>G	p.Met79Val	3	VUS	Benign	BA1	NA	NA	NA
c.247G>A	p.Gly83Ser	3	Conflicting	Likely Benign	BS1, BP4_P	NA	NA	NA
c.263C>T	p.Pro88Leu	3	Conflicting	Benign	BA1, BP4_P	NA	NA	NA
c.403C>G	p.Pro135Ala	4	Conflicting	Benign	BA1, BS2	NA	NA	NA
c.407C>T	p.Ala136Val	4	Conflicting	Benign	BA1, BP4_P	NA	NA	NA
c.425G>T	p.Gly142Val	4	Conflicting	Benign	BA1	NA	NA	NA
c.442G>A	p.Gly148Arg	4	Conflicting	Benign	BA1, BS2_P	NA	NA	NA
c.458C>T	p.Pro153Leu	4	VUS	Likely Benign	BS1	NA	NA	NA
c.508A>T	p.Thr170Ser	4	VUS	Likely Benign	BS1, BP4_P	NA	NA	NA
c.655A>G	p.Thr219Ala	4	Conflicting	Benign	BA1, BP4_P	NA	NA	NA
c.665C>T	p.Pro222Leu	4	Conflicting	Likely Benign	BS1, BS2_P	NA	NA	NA
c.685GGMCCM[n]	p.229_230GP[n]	4	VUS	Benign	BA1, BS2, BP3	NA	NA	NA
c.727G>A	p.Gly243Ser	4	Conflicting	Benign	BA1	NA	NA	NA
c.802G>A	p.Val268Met	5	Conflicting	Benign	BA1, BP4_P	NA	NA	NA
c.811G>A	p.Gly271Arg	5	Conflicting	Likely Benign	BS1	NA	NA	NA

c.914C>T	p.Pro305Leu	6	Conflicting	Benign	BA1	NA	NA	NA
c.952G>A	p.Val318Ile	6	Conflicting	Benign	BA1	NA	NA	NA
c.1013A>G	p.Gln338Arg	6	Conflicting	Likely Benign	BS1	NA	NA	NA
c.1018G>A	p.Ala340Thr	6	Conflicting	Benign	BA1, BP4_M	NA	NA	NA
c.1076G>A	p.Arg359Gln	6	Conflicting	Benign	BA1, BS2, BP4_P	NA	NA	NA
c.1098C>G	p.Ile366Met	6	Conflicting	Likely Benign	BS1	NA	NA	NA
c.1847C>T	p.Pro616Leu	12	Conflicting	Benign	BA1	NA	NA	NA
c.2021C>T	p.Pro674Leu	14	Conflicting	Benign	BA1, BP4_P	NA	NA	NA
c.2066A>T	p.Lys689Met	14	VUS	Likely Benign	BS1	NA	NA	NA
c.2176C>T	p.Arg726Cys	15	VUS	Likely Benign	BS1	NA	NA	NA
c.3358G>A	p.Gly1120Ser	24	Conflicting	Benign	BA1	NA	NA	NA
c.3436G>A	p.Gly1146Ser	25	Conflicting	Benign	BP4_M, BA1	NA	NA	NA
c.3791C>T	p.Thr1264Met	27	Conflicting	Benign	BA1, BP4_M	NA	NA	NA
c.3830C>T	p.Pro1277Leu	27	Conflicting	Benign	BA1	NA	NA	NA
c.3841G>A	p.Val1281Ile	27	Conflicting	Likely Benign	BS1, BP4_M	NA	NA	NA
c.4211T>G	p.Val1404Gly	30	Conflicting	Benign	BA1, BS2, BP4_P	NA	NA	NA
c.4351G>A	p.Ala1451Thr	30	Conflicting	Benign	BA1, BP4_P	NA	NA	NA
c.4501G>A	p.Val1501Met	31	VUS	Likely Benign	BS1, BP4_P	NA	NA	NA
Variants re-classified as VUS/Likely pathogenic								
c.482C>T	p.Ala161Val	4	Not in ClinVar	VUS	BP4_M, PS4_M, BP1	22	SCCOHT	PMID: 26942101
c.2335G>A	p.Asp779Asn	16	VUS	Likely Pathogenic	PP3_S, PS4_S, PM2_P	1.3, 30	ATRT, SCCOHT	PMID: 25060813, 24658002
c.2375T>C	p.Leu792Pro	16	Not in ClinVar	Likely Pathogenic	PP3_S, PS4_S, PM2_P	23, 29	SCCOHT	PMID: 27866340
c.2573C>A	p.Thr858Lys	18	Not in ClinVar	VUS	PP3_M, PM2_P, PS4_M	NA	SCCOHT	PMID: 26325560
c.2783T>C	p.Leu928Pro	19	VUS	Likely Pathogenic	PP3_S, PS4_M, PM2_P	26	SCCOHT	PMID: 24658001

c.2915T>C	p.Leu972Pro	20	Not in ClinVar	Likely Pathogenic	PP3_S, PS4_M, PM2_P	18	SCCOHT	PMID: 24658002
c.3146C>T	p.Pro1049Leu	22	Not in ClinVar	VUS	PP3_M, PS4_P, PM2_P	1,5	MRT	NA
c.3239G>A	p.Gly1080Asp	24	Pathogenic	Likely Pathogenic	PP3_S, PS4_S, PM2_P	13, 40, 39	SCCOHT	PMID: 24658002, Internal data
c.3241A>G	p.Lys1081Glu	24	Not in ClinVar	VUS	PP3_M, PS4_M, PM2_P	26	SCCOHT	PMID: 26343384
c.3302T>C	p.Leu1101Pro	24	Not in ClinVar	VUS	PP3_M, PS4_M, PM2_P	16	SCCOHT	NA
c.3541C>T	p.His1181Tyr	25	Not in ClinVar	VUS	PP3_M, PS4_M, PM2_P	27	SCCOHT	NA
c.3574C>T	p.Arg1192Cys	26	VUS	Likely Pathogenic	PP3_S, PS4_M	3,8	MRT	PMID: 26343384
c.3774+2T>A	NA	27	Likely Pathogenic	VUS	PM2_P	29, NA	Unknown, Neuroblastoma	NA
c.4170G>C	p.Lys1390Asn	29	Not in ClinVar	Likely Pathogenic	PS4_M, PM2_P, PP3_P, PS1_M	0-2	MRT	PMID: 33020650
c.4170G>A	p.Lys1390Lys	29	Not in ClinVar	Likely Pathogenic	PS4_M, PM2_P, PP3_P, PS1_M	1	ATRT	PMID: 34185381
c.4180_4181delinsC	p.Gly1394fs	30	VUS	VUS	PM2_P	57, NA	Epithelial ovarian cancer, Unaffected	PMID: 29204511
c.4208del	p.Ser1403fs	30	VUS	VUS	PM2_P	34	DCIS	NA
c.4226_4227insATTC	p.?	30	Not in ClinVar	VUS	PM2_P	5	Progressive distal motor neuropathy, suspected Charcot-Marie-Tooth disease.	NA
c.4266+1G>C	NA	30	VUS	VUS	PM2_P	44	Myxofibrosarcoma	NA
c.4266+1G>T	NA	30	VUS	VUS	No criteria met	NA, 46	Unaffected, Invasive ductal carcinoma +/+/+	NA
c.4266+2T>C	NA	30	VUS	VUS	PM2_P	46	Colorectal cancer	NA
Exon 30 deletion	NA	30	VUS	VUS	PM2_P	50s	Unaffected	NA
Exon 30 deletion	NA	30	VUS	VUS	PM2_P	50s	Breast cancer	NA
Exon 30 deletion	NA	30	VUS	VUS	PM2_P	40s	Breast cancer	NA

Exon 30 deletion	NA	30	VUS	VUS	PM2_P	40s	Ovarian cancer (BRCA1 variant found)	NA
Exon 30 deletion	NA	30	VUS	VUS	PM2_P	30s	Breast cancer (BRCA2 variant found)	NA
Exon 30 deletion	NA	30	VUS	VUS	PM2_P	40s	Breast cancer	NA
Exon 30 deletion	NA	30	VUS	VUS	PM2_P	50s	Breast cancer	NA
Exon 30 deletion	NA	30	VUS	VUS	PM2_P	30s	Kidney cancer	NA

NA, Not available; VUS, Variant of Uncertain Significance; SCCOHT, Small-cell carcinoma of the ovary, hypercalcemic type; ATRT, atypical/typical rhabdoid tumor; MRT, malignant rhabdoid tumor; DCIS, Ductal carcinoma in situ.

3.2. Molecular Analysis of Splicing Variants

The objective of this aim was to use functional assays where samples were available to investigate putative splice variants. While splicing prediction tools exist, they have generally been less reliable at predicting pathogenicity than tools used for missense variants, making RNA-based validation the gold standard for classification of splicing variants.

Through our laboratory Biobank, we identified five probands with non-*SMARCA4*-related phenotypes who carried the c.3951+2T>C splice variant in exon 28 on *SMARCA4* transcript NM_001128849.3. The adjacent c.3951+1G>A variant at this splice site was seen in a woman with SCCOHT. This variant has been classified as likely pathogenic by several laboratories.

cDNA analysis and agarose gel electrophoresis of cDNA amplification products from three different samples (sample 1: proband with the c.3951+2T>C variant, sample 2: negative (non-carrier) aunt as control, sample 3: SCCOHT patient with the c.3951+1G>A variant) revealed two bands at 878 and 779 bp (**Figure 10**). Agarose gel Sanger sequencing of cDNA showed that the lower bands, found identical in all samples, included exons 26, 28, 29, and 30 (not shown in Figure 11) corresponding to the 779 bp band (**Figure 11B, D, F, H, J and L)**. Sequencing of the upper bands revealed retention of a part of intron 28 in samples 1 and 3 (treated with cycloheximide) (**Figure 11A and I)**. The retained portion of intron 28 was an in-frame 99-nucleotide long sequence:

CTGCTAGGGATACCACCATGGGCACTAGGACGTCTGCTTTGCTCCTGGTGGCAGC

AGAGTGGGATGCCTGAACTGCCCCATCCAGCCTGCAGCGCTTAC.

This in-frame retention produced a premature stop codon, which could subject the transcript to NMD, potentially leading to partial or complete loss of the protein. However, band patterns did not change with cycloheximide treatment, suggesting that NMD is not occurring and that

the splicing defect is inefficient, allowing for sufficient expression of the wild-type protein product. Sanger sequencing of the upper bands showed the expression of exons 26 and 27, 28, 29, and 30 (not shown in Figure 11) in samples 1 (without cycloheximide), 2, and 3 (without cycloheximide) (**Figure 11C, E, G, and K)**. Despite conducting cDNA analysis of LCLs, the exact mechanisms of action remain unclear. The samples, along with controls, will undergo in depth RNA sequencing Analysis in collaboration with Dr. Barbara Rivera Polo's laboratory (IDIBELL, Spain) to further elucidate the underlying molecular mechanisms.

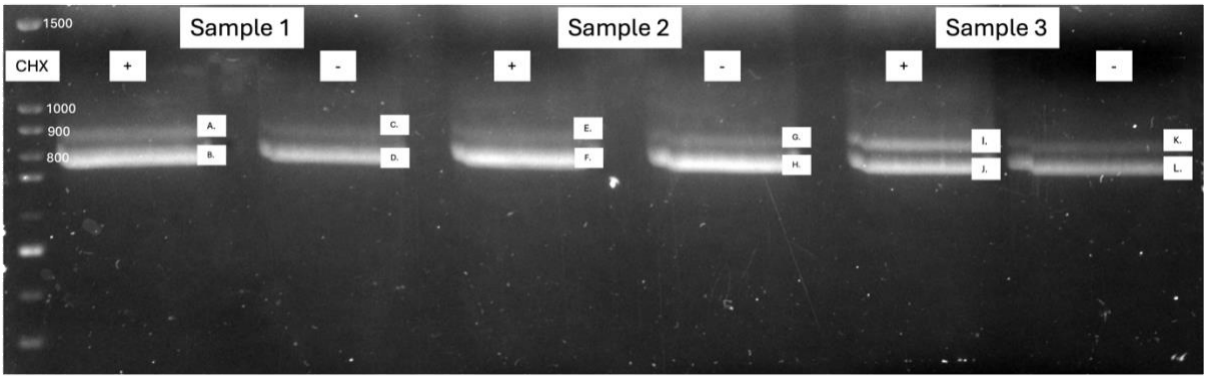
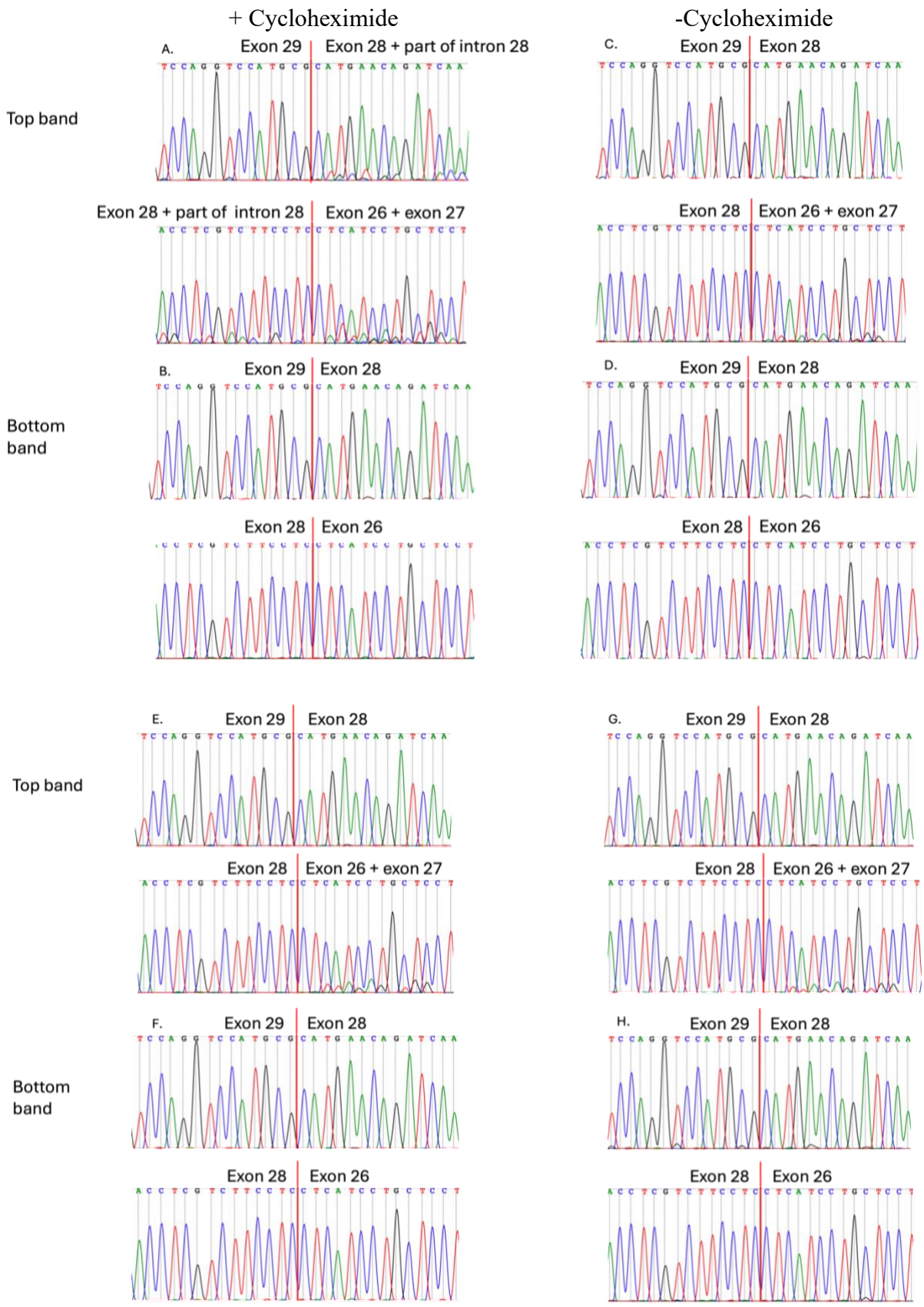


Figure 10. Agarose gel of cDNA amplification of the splicing variants of the c.3951+2T>C variant, the negative control, and the c.3951+1G>A variant. CHX = +/- cycloheximide treatment. Letters correspond to sequencing of bands in Figure 11.


+ Cycloheximide
-Cycloheximide
Top band
Bottom band
A.
Exon 29
Exon 28 + part of intron 28
C.
Exon 29
Exon 28
Exon 28 + part of intron 28
Exon 26 + exon 27
Exon 28
Exon 26 + exon 27
B.
Exon 29
Exon 28
D.
Exon 29
Exon 28
Exon 28
Exon 26
Exon 28
Exon 26
Top band
E.
Exon 29
Exon 28
G.
Exon 29
Exon 28
Exon 28
Exon 26 + exon 27
Exon 28
Exon 26 + exon 27
Bottom band
F.
Exon 29
Exon 28
H.
Exon 29
Exon 28
Exon 28
Exon 26
Exon 28
Exon 26

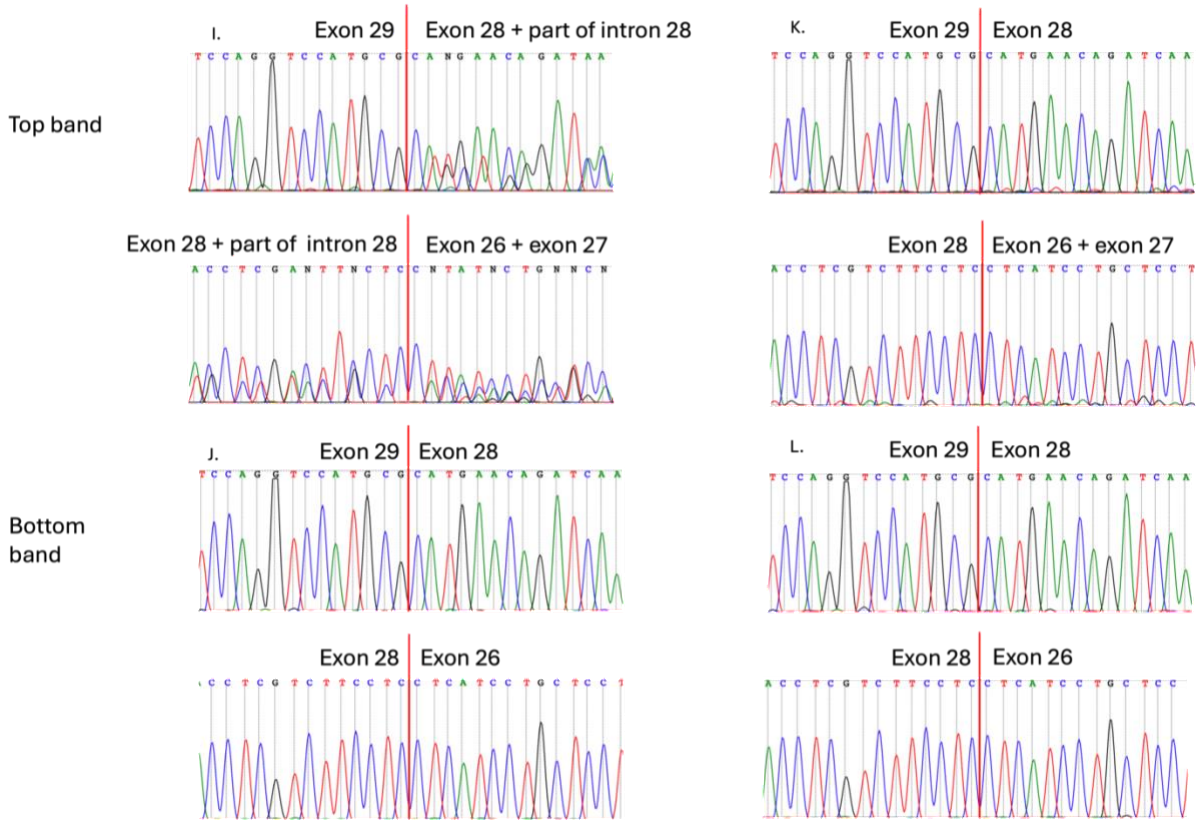


Figure 11. Sanger sequencing of cDNA amplification of the c.3951+2T>C and c.3951+1G>A variant. Reverse primer was used. (A-D) show sequencing data of Sample 1 (c.3951+2T>C carrier). (A+B) show sample treatment with cycloheximide, (C+D) show sample without treatment. (A+C) show top bands on the gel. (B+D) show bottom bands on the gel. Sanger sequencing data shows retention of a part of intron 28 in the top band of c.3951+2T>C carrier, only present in the sample treated with cycloheximide. Sample without cycloheximide does not show this retention. (E-H) show sequencing data of Sample 2 (negative control). (E+F) show sample treatment with cycloheximide, (G+H) show sample without treatment. (E+G) show top bands on the gel. (F+H) show bottom bands on the gel. Sanger sequencing data shows no retention of a part of intron 28. (I-D) show sequencing data of Sample 3 (c.3951+1G>A SCCOHT patient). (I+J) show sample treatment with cycloheximide, (K+L) show sample without treatment. (I+K) show top bands on the gel. (J+L) show bottom bands on the gel. Sanger sequencing data shows retention of a part of intron 28 in the top band

of c.3951+1G>A SCCOHT patient, only present in the sample treated with cycloheximide. Sample without cycloheximide does not show this retention.

3.3. Using Functional Assays to determine the impact of *SMARCA4* variants.

The objective of this aim was to reproduce optimization experiments previously conducted by another trainee working under the supervision of collaborator Dr Sidong Huang. The purpose of the assay is simply to assess the impact of expressing various *SMARCA4* variants, using variants known to be benign, causing CCS, causing ATRT or causing SCCOHT as a reference. The reason for reproducing the assay was that the original experiments used commercially prepared variant constructs that were later found to contain an unwanted genetic change in the *SMARCA4* sequence. While it was deemed unlikely that this change would affect protein function, to rule out any potential confounding effect by this change, new constructs were generated and were used to repeat the experiments. Once the assay is optimized with the correct constructs, it can be used to test and classify variants of unknown significance in *SMARCA4* for their pathogenic potential.

To determine the impact of selected variants on the behaviour of the SCCOHT-1 cell line, established functional assays were used. After successful lentiviral transduction and infection, the cells were be collected for each variant, as explained in sections 2.5.2 and 2.5.4.

Protein lysates were gathered to gauge protein expression. Prior work cited above indicates that cyclin D1 expression in SCCOHT cells will not be upregulated by *SMARCA4* PVs.[32] In contrast, variants that are likely not affecting SMARCA4 function or not pathogenic in the context of SCCOHT will restore cyclin D1 expression. Expression of other proteins mentioned in section 2.5.3. were also investigated, including BRG1, BRM, IP3R3 and CD44.

We also used colony formation and cell viability assays to investigate how *SMARCA4* variants affect growth of SCCOHT cells. In contrast to non-pathogenic variants, as previously established,[32] we expect that forced expression of PVs will fail to suppress SCCOHT growth.

Preliminary results:

Short-term cell viability assay indicated that CSS-related and benign variants (C1, C2, B, WT) significantly suppressed SCCOHT-1 cell growth, leading to cell death within 5 days (**Figure 12**). Notably, SCCOHT-related non-truncating variants (S1, S3) showed less suppression, similar to the empty vector (control), except for the S2 which displayed reduced viability (**Figure 12)**. ATRT-related (A1) and the ATPase dead (ATP) variants resulted in approximately 30% cell survival.

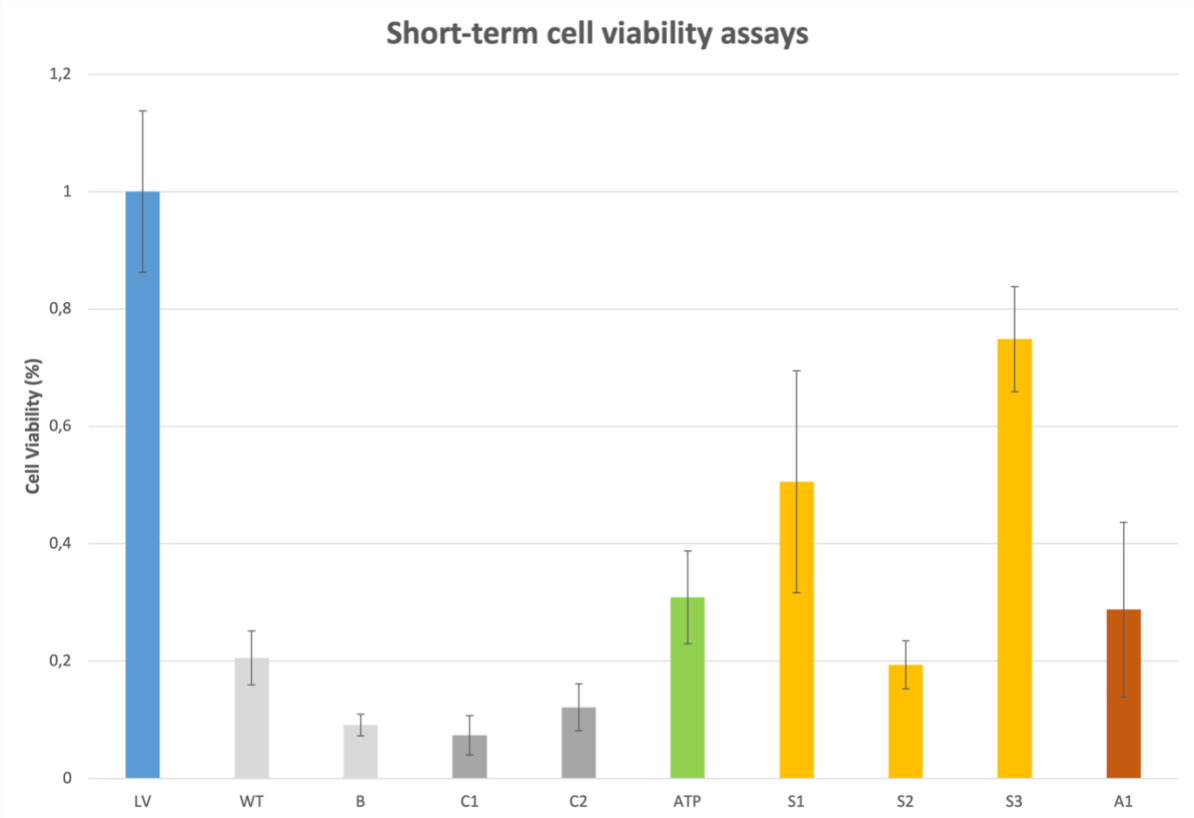


Figure 12. Short-term cell viability assay in SCCOHT-1 cells. The bar graph shows the cell viability percentages for different variants introduced: Lv (Control), WT (Wild Type), B (Benign variant), C1 (non-truncating variant causing CSS), C2 (non-truncating variant causing CSS), ATP (ATPase dead variant), S1 (non-truncating variant causing SCCOHT), S2 (non-truncating variant causing SCCOHT), S3 (non-truncating variant causing SCCOHT), and A1 (non-truncating variant causing ATRT). The y-axis represents the percentage of cell viability,

with error bars indicating standard deviation from the mean. Lv exhibits the highest viability, set as the reference (100%). Among the variants, SCCOHT variants show the highest viability, along with the ATRT variant, except for S2 variant. Notably, the ATPase dead variant does not lead to cell death as expected. The other variants, WT, B, C1 and C2 show significantly lower viability compared to the control.

The preliminary observations shown here are consistent with results that were obtained using the previous constructs. Notably, the SCCOHT-related variants exhibit higher cell viability compared to both the other variants and the control. The cell viability of S2 should however be higher. Conversely, the benign, wild-type, and CSS-related variants demonstrate elevated cell mortality, aligning with previously obtained results. Additionally, the ATPase dead variants, similar to the ATRT variant, show reduced, yet still higher than controls, cell viability, which corroborates prior findings. However, replicate experiments yielded variable results, so it is necessary to keep repeating the experiments until consistent observations are obtained, or until we can identify the source of the variability. At the time of writing this thesis, these experimental results remain preliminary, and the work is ongoing. In the Appendix, Section 2, final results of short- and long-term cell viability assays are presented, alongside the results of completed protein expression assays.

3.4. Exploring DNA methylation profiling as a potential biomarker for *SMARCA4* variants

The objective of this aim was to investigate the potential for using methylation patterns as a biomarker by comparing DNA methylation profiles associated with variants known to cause CCS vs SCCOHT, looking for disease-specific patterns. The experiments could not be

completed in time for submission of this thesis because of some missing controls, but the work I performed so far is described here.

The ideal design for this experiment would include samples bearing every type of variant that could be encountered in patients with SCCOHT or CCS. The SCCOHT-1 cell constructs designed for the cell survival assays described in section 3.3 meet this requirement. Thus, we collected 12 samples from our SCCOHT-1 cell line, including the 11 variants specified in **Table 5**, along with a sample of untreated SCCOHT-1 cells.

However, because these variant constructs are expressed in a SCCOHT line derived from a patient tumor, it is possible that the methylation patterns observed in these samples will reflect the tumor background of the cells rather than the specific variants being expressed from the constructs. In addition, variants expressed in the cell line are effectively homozygous, while in patient samples such as blood, the germline variants will exist in the heterozygous state, co-existing with a normal copy of the gene. Therefore, we also included a second arm to this experiment consisting of DNA samples from patient blood.

We collected samples from 20 patients, categorized into five groups based on specific criteria: (1) individuals without *SMARCA4* variants who do not have SCCOHT, (2) individuals with *SMARCA4* germline variants who do not have SCCOHT, (3) germline-negative SCCOHT patients, (4) SCCOHT patients with truncating *SMARCA4* variants, and (5) SCCOHT patients with non-truncating *SMARCA4* variants (**Table 6**). Notably, patients in the fifth group harbor *SMARCA4* variants that are also represented in the SCCOHT-1 cell line variants, allowing for cross-validation. To avoid sex-based differences in DNA methylation signatures, all patients were female. Furthermore, to eliminate age-related variations in DNA methylation, samples were age-matched across different groups. Control individuals without SCCOHT were required to be over the age of 50 to ensure they were unlikely to develop SCCOHT later in life.

Table 5. DNA samples from non-truncating *SMARCA4* variants in SCCOHT-1 cell line.

Variant	Variant Type	Phenotype
c.3380A>G	Missense	CSS
c.1636_1638del	In-frame deletion	CSS
c.3610_3615del	In-frame deletion	SCCOHT
c.2491_2505del	In-frame deletion	ATRT
c.2335G>A	Missense	ATRT
c.3239G>A	Missense	SCCOHT
c.2311_2316del	In-frame deletion	SCCOHT
c.1114T>C	Missense	Benign
c.2354A>G	Missense	ATPase dead
Wildtype	NA	Control
Empty vector	NA	Control
Unaltered cells	NA	SCCOHT

NA, Not available; SCCOHT, Small-cell carcinoma of the ovary, hypercalcemic type; ATRT, atypical/typical rhabdoid tumor; CSS, Coffin-Siris Syndrome

Table 6. DNA samples from patients.

Age at blood drawn	*SMARCA4* variant	Treatment	Status
Without variant without disease			
41	Negative	NA	Acquired
48	Negative	NA	Acquired
50	Negative	NA	Acquired
70	Negative	NA	Acquired
With variant without disease			
55	Germline: c.1921dup (p.Ala641Glyfs*10)	NA	Acquired
70	Germline: c.133_134delinsA	NA	Acquired
? (female +50)	?	NA	Not Acquired
? (female +50)	?	NA	Not Acquired
Germline negative SCCOHT patients			
12	Somatic: c.2438+1 G>A	NA	Acquired
29	Somatic: c.2859+1 G>A; c.4170+2 T> G	6 cycles VPCBAE	Acquired
41	Somatic: c.3997_3998insGGAAG	Cisplatine/Etoposide	Acquired
51	Somatic: c.2438+1G>A; c.3982del	3 cycles cisplatin & etopiside, 7 cycles of vac/ice, Taxol	Acquired

SCCOHT patient with truncating *SMARCA4* variants			
20	Germline: c.1921dup (p.Ala641Glyfs*10)	6 cycles PAVEP	Acquired
24	Germline: c.2527del (p.Ala843Profs*15), Somatic: c.2527del (p.Ala843fs)	4 cycles cisplatin, etoposide, bleomycin	Acquired
24	Germline: c.917_941del, p.Gln306Argfs*12	6 cycles VPCBAE	Acquired
29	Germline: c.3229C>T (p.Arg1077*)	5 cycles VPCBAE	Acquired
SCCOHT patient with non-truncating *SMARCA4* variants			
Daughter- NA: diagnosed at 13, recurrence at 19	c.3239G>A	NA	Acquired
Mother – NA: diagnosed at 40	c.3239G>A	NA	Acquired
68	c.3951+1G>A	NA	Acquired
36	c.3951+1G>A	NA	Acquired

NA: Not applicable; VPCBAE, vinblastine, cisplatin, cyclophosphamide, bleomycin, doxorubicin, etoposide; vac/ice, Vincristine, Adriamycin, Carboplatin/Ifofsomide, Cisplatin, Etoposide; PAVEP, Adriamycin, Cisplatin, Cyclophosphamide, Etoposide.

As proposed by Aref-Eshghi et al.,[77] genomic DNA (gDNA) must be extracted from all samples for DNA methylation analysis. We obtained gDNA from the cell line samples through lentiviral transduction and infection of SCCOHT-1 cells, as detailed in Sections 2.5.2 and 2.5.3, followed by gDNA extraction. This process was challenging due to the concentration and volume requirements for the samples for DNA methylation analysis, making it difficult to obtain enough gDNA. gDNA from patient blood samples was obtained from our laboratory's Biobank and SCCOHT registry. However, some control samples are still lacking.

The rarity of the disease and the infrequency of non-truncating variants causing SCCOHT hindered our ability to include a diverse range of SCCOHT patients with non-truncating *SMARCA4* variants. Consequently, we duplicated two variants: the c.3239G>A variant, observed in a mother and daughter, and the c.3951+1G>A variant, found in two patients. This will also avoid potential age-related differences in DNA methylation signatures.

Furthermore, we still lack samples from unaffected females over the age of 50 with a *SMARCA4* variant. Although our SCCOHT/SMARCA4 registry facilitates sample collection, these samples are often from SCCOHT patients and their relatives, making the missing samples particularly difficult to obtain. We are currently awaiting additional recruitment from the Registry/Biobank to complete the sample set necessary for conducting the experiments, a task which extends beyond the timeframe of this thesis.

4. Discussion

4.1. Contributions to knowledge from this thesis

SCCOHT is a genetic disease, caused by inherited or somatic pathogenic variants in *SMARCA4*. Correctly distinguishing variants that are pathogenic from those that are not pathogenic for SCCOHT is therefore central to patient management. Effective treatment of SCCOHT remains an unmet clinical challenge, as SCCOHT is highly resistant to conventional chemotherapy and lacks effective alternative options. The biological mechanisms by which *SMARCA4* variants drive SCCOHT are currently unknown, especially given our limited understanding of why certain missense variants cause CSS while others result in cancer. The main risk-reducing intervention on unaffected females carrying pathogenic variants is preventive oophorectomy, which has been performed on females as young as 6 years old, with life-changing consequences. [28] Given all these challenges, it is urgent to advance our understanding of *SMARCA4* variants to better inform risk assessment and improve clinical outcomes. Telling a family that their teenage daughter must have her ovaries removed requires that we be certain the variant she carries puts her at risk for SCCOHT while, conversely, we must be certain that the variant a patient carries, considered to be a VUS, is not in fact a LP/P variant, which would mean that the patient not undergoing preventive oophorectomy may develop SCCOHT. The accurate classification of variants in *SMARCA4* impacts risk assessment for all *SMARCA4*-related tumors including SCCOHT, ATRT and MRT. In Aim 1 of this thesis, we performed an in-depth literature review, integrating data from multiple sources, and applying modified ACMG/AMP criteria, we were able to confidently reclassify 69 non-truncating *SMARCA4* variants, providing clinically meaningful insights into the pathogenicity or benignity of these genetic changes. Furthermore, we classified 64 truncating variants that were seen in previously unpublished patients (**Supplementary Table S1**). We

propose 11 adaptations of the ACMG/AMP criteria. There is a total of 27 criteria; 11 were modified, 7 were not applicable and 9 remained unmodified.

The current PVS1 criterion from Richards et al.[51] is "null variant (nonsense, frameshift, canonical ±1 or 2 splice sites, initiation codon, single or multi exon deletion) in a gene where LOF is a known mechanism of disease". However, based on cDNA sequencing and *in silico* tools, we suggest that this criterion should be adapted and not applied to variants in exon 27 and 30 in transcript NM_001128849.3, as they are unlikely to predispose individuals to SCCOHT due to their lack of expression in the ovary. The current MANE select transcript is NM_003072, which does not include exon 30, and therefore any variants previously classified as likely pathogenic in this exon would be annotated as deep intronic variants with the MANE select transcript.[78] However, the MANE select plus clinical transcript does include exon 30. Furthermore, we implemented the PVS1 (RNA) criterion for splicing variants, as recommended by Walker et al.[70] For purposes of application of the PVS1 code for splicing variants, the ATPase domain (exons 16-25) is considered a critical domain. At present, there is a notable absence of well-established functional assays tailored for *SMARCA4* variants that are associated with SCCOHT, ATRTs and MRTs, hindering the ability to provide strong evidence on the effect of a variant on gene or protein function. Functional studies are particularly challenging due to SCCOHT's aggressive nature and complexities related to cell viability after the loss or the reinsertion of SMARCA4 in SCCOHT cell lines. Therefore, we propose applying PS3 at a supporting level when an *in vitro* study demonstrates that the variant leads to loss or decreased SMARCA4 expression, and the study meets the criteria specified in Brnich et al.[72]

We adapted PS4 to reflect the rarity of SCCOHT, MRT, and ATRT, with varying levels of evidence based on the number of probands affected (**Table 7**). This adjustment acknowledges the challenges of conducting odds ratio calculations for these rare tumors and emphasizes the

need for gene-specific criteria in variant classification. Moreover, the PS4 criterion was also tailored to *SMARCA4's* role as a defining gene for SCCOHT, MRT, and ATRT, by considering loss of expression in the tumor, the presence of a second pathogenic variant, or loss of heterozygosity (LOH), as proposed by Walsh et al.[79] SCCOHT tumors are relatively quiescent in terms of tumor variant burden and apart from extremely rare reports of GPVs in *SMARCB1*,[9,10] there appear to be no other causative genes for SCCOHT. Furthermore, the mechanism of disease of the germline and somatic variants is not expected to differ, with both expected to cause loss of function. Therefore, if the *SMARCA4* variant in question is found in conjunction with another pathogenic variant or LOH in a SCCOHT tumor, but the provenance of the variant is unknown or found to be somatic, this code should be applied.

Importantly, the prevalence of SCCOHT remains unknown, making it challenging to determine the significance of individual variants within *SMARCA4*. Therefore, it was approximated to be 1/1,000,000, with a penetrance of SMARCA4 of 20%.[76] As there are no established data on the penetrance of *SMARCA4* variants in SCCOHT, the use of the BS2 criterion, a strong evidence code for benignity, was based on a conservative estimation of penetrance. The lack of pathogenic variants in the gnomAD database, which suggested a possible high penetrance rate, provided additional support for this strategy. To date, we are not aware of any individual with homozygosity for pathogenic *SMARCA4* variants. In light of the presumed embryonic lethality of homozygous *SMARCA4* variants, the BS2 criterion was adapted to include the detection of *SMARCA4* variants in homozygous individuals. It is crucial for accurate classification, that collective effort is undertaken in determining the exact prevalence and penetrance of SCCOHT and *SMARCA4*.

For the BP1 code, we applied it as per ACMG/SVI guidelines, except for variants in the ATPase domain (residues 766 to 1246), as there are no known pathogenic missense variants outside this region. The BP3 criterion relating to in-frame insertions and deletions in repetitive

regions is applicable to in-frame insertions and deletions between p.Gly229 and p.Pro244. Twelve Gly-Pro repeats are found in this area, and the presence of >200 alleles in gnomAD v4.1 with in-frame insertions and deletions in this region allowed us to classify these variants as likely benign.

Table 7. Modification of PS4 code with a point system.

Applies only to patients with SCCOHT/MRT/ATRT.

	Germline	**Somatic**
No tumor sequencing OR SMARCA4 expression retained by IHC	1 point	1 point
Second LP/P variant or LOH **SMARCA4 expression must be absent in tumor	2 points	2 points†

†Not applicable if 2 additional LP/P variants are detected. Points are additive across tumors/probands. NA, Not Applicable.

Strong	**Moderate**	**Supporting**
4+ points	2-3 points	1 point

Add points to obtain classification.

4.2. Limitations

While Aim1 of this thesis provides a comprehensive framework for variant classification in the context of *SMARCA4*-related cancers, several limitations should be noted. The rarity of SCCOHT poses challenges in gathering sufficient clinical and functional data for variant interpretation. Conflicting results are common for missense variants in *SMARCA4* and represent a substantial challenge for variant classification. Future studies should continue to collect data to validate our findings and refine the proposed classification criteria. Moreover,

functional studies validating the impact of specific variants on SMARCA4 function and tumor development would strengthen the evidence for variant pathogenicity.

Analysis of splicing variants performed in Aim 2 indicated that the c.3951+2T>C and c.3951+1G>A variants in *SMARCA4* lead to the retention of part of intron 28, introducing a premature stop codon but not resulting in significant NMD, suggesting a potential mechanism for the partial preservation of SMARCA4 function in these variants. However, this splicing defect was inefficient, allowing for the production of potentially sufficient wild-type protein to meet biological function requirements. Despite conducting cDNA analysis of LCLs, the exact mechanisms of action remain unclear. The samples, along with controls, will undergo in depth RNA sequencing analysis in collaboration with Dr. Barbara Rivera Polo's laboratory (IDIBELL, Spain) to further elucidate the underlying molecular mechanisms.

In Aim 3, we sought to determine the impact of *SMARCA4* variants on the behaviour of the SCCOHT-1 cell line by using established assays, to determine the pathogenic potential of these variants. However, due to reproducibility issues with the new construct and the need for protocol refinement, this part of my project could not be completed within the timeframe of my thesis. Once optimized, these cell viability and colonly formation assays will be performed to investigate how the various *SMARCA4* variants affect growth of SCCOHT cells. Protein expression assays will also be conducted, by investigating the expression of cyclin D1, BRM, BRG1, CD44 and IP3R3 expression. Cyclin D1 is a regulatory subunit of cyclin-dependent kinases CDK4 and CDK6. BRM (SMARCA2) and BRG1 (SMARCA4) are ATPase subunits of SWI/SNF involved in SCCOHT. CD44 is a cell surface adhesion receptor that is highly expressed in many cancers and regulates metastasis via recruitment of CD44 to the cell surface.[68] Lastly, IP3R3 is a protein encoded by the ITPR3 gene, releasing calcium ion (Ca2+)

from the endoplasmic reticulum (ER) into the cytosol, and is involved in chemotherapy resistance.[33]

Similarly, for the methylation profiling, the strict requirements of the experimental design needed to generate interpretable results and the rarity of the disease meant we were unable to obtain all the required samples and controls to execute this experiment in time for completion of this thesis. We are missing samples from unaffected females over the age of 50 with a *SMARCA4* variant, as well as supplementary samples from patients harbouring non-truncating *SMARCA4* variants. Younger unaffected females still have a risk of developing the disease and thus would not be a good control, and performing the experiment without all the required controls would likely lead to uninterpretable results. We are hopeful that we can eventually collect these samples via our own SCCOHT/SMARCA4 registry, but it may take time. The missing samples are the rarest observed, partly due to what we believe to be high penetrance of the variants, and partly because there may be biases with regards to which patients are more likely to be motivated to participate in the SCCOHT/SMARCA4 Registry and Biobank.

5. Conclusions and Future directions

In conclusion, the work from this thesis produced a comprehensive improved framework for variant classification in the context of SMARCA4-related cancers. By refining ACMG/AMP criteria, we have confidently reclassified 69 non-truncating variants. We also report classification of 64 truncating variants seen in 73 previously unpublished patients with SCCOHT, MRT, or ATRT. These refined criteria address the critical need for more accurate variant classification and will reduce the number of VUSs reported. This advancement not only enhances the accuracy of genetic testing but also has profound implications for patient management and genetic counseling. Improved classification of *SMARCA4* variants allows for personalised and targeted risk assessment, which leads to improved clinical outcomes. It allows for the identification of at-risk individuals allowing for timely preventive measures. The implications of our findings are thus far-reaching. Furthermore, our study underscores the importance of continued research and collaboration in the field of genetic variant classification. By sharing data and refining classification criteria, we can collectively enhance our understanding of *SMARCA4*-related disorders and improve the accuracy of genetic diagnoses. Future studies should continue to collect data to validate our findings and continue to refine the proposed classification criteria. At the same time, optimization of cell survival assays and methylation profiling as potential tools to perform variant classification will build on the preliminary work I performed and continue to be developed in my lab. These promising assays will provide essential data to validate our findings and refine the proposed classification criteria. These studies will elucidate how various *SMARCA4* variants impact SCCOHT cell growth and protein expression, and how DNA methylation signatures differ between different *SMARCA4* variants, contributing to a better understanding of SMARCA4's role in disease pathology. Collectively, these efforts will enhance diagnostic accuracy and significantly improve patient management.

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Appendix

1. Supplementary Figures and tables

Table S1. Previously unpublished cases of RTPS tumors.

Case ID	Exon/Intron	cDNA	Protein	Variant type	Origin of variant	Tumor type	Classification (Codes applied)	Source
1[1]	1	NC_000019.9:g.(?_11053907)_(11083913_?)	Exon 1 deletion	Deletion	Germline	SCCOHT	Pathogenic(PVS1,PS4_P,PM2_P)	Testing lab
2	2	c.133_134delinsA	p.Pro45Thrfs*49	Frameshift	Germline	SCCOHT	Pathogenic(PVS1,PS4_P,PM2_P)	Registry
3	3	c.227dup	p.Met76Ilefs*6	Frameshift	Germline	SCCOHT	Pathogenic(PVS1,PS4_P,PM2_P)	Testing lab
4	4	c.430C>T	p.Gln144Ter	Nonsense	Germline	SCCOHT	Pathogenic(PVS1,PS4_P,PM2_P)	Testing lab
5,6	4	c.493C>T	p.Gln165Ter	Nonsense	Unknown (x1), Germline (x1)	SCCOHT (x2)	Pathogenic(PVS1,PS4_M,PM2_P)	Personal communication
7	4	c.613C>T	p.Gln205Ter	Nonsense	Germline	SCCOHT	Pathogenic(PVS1,PS4_P,PM2_P)	Personal communication
8	5	c.788dup	p.Gly264Argfs*23	Frameshift	Germline	RTPS2	Pathogenic(PVS1,PS4_P,PM2_P)	Testing lab
9	5	c.797C>A	p.Ser266Ter	Nonsense	Somatic	SCCOHT	Pathogenic(PVS1,PS4_P,PM2_P)	Personal communication
10	5	c.826_827del	p.Pro276Serfs*10	Frameshift	Germline	SCCOHT	Pathogenic(PVS1,PS4_P,PM2_P)	Poster presentation
11	5	c.849del	p.Trp284Glyfs*19	Frameshift	Germline	SCCOHT	Pathogenic(PVS1,PS4_P,PM2_P)	Registry
12	6	c.917_941del	p.Gln306Argfs*12	Frameshift	Germline	SCCOHT	Pathogenic(PVS1,PS4_P,PM2_P)	Registry
13	7	c.1167del	p.Ser391Profs*20	Frameshift	Somatic	SCCOHT	Pathogenic(PVS1,PS4_P,PM2_P)	Registry
14,15	7	c.1141C>T	p.Arg381Ter	Nonsense	Germline (x2)	Non-specific	Pathogenic(PVS1,PS4_S)	Poster presentation

						ovarian, SCCOHT		
16	7	c.1183del	p.Asp395Ilefs*16	Frameshift	Germline	SCCOHT	Pathogenic(PVS1,PS4_P,PM2_P)	Poster presentation
17,18,19,20	7	c.1189C>T	p.Arg397Ter	Nonsense	Unknown (x1), Germline (x3)	SCCOHT (x4)	Pathogenic(PVS1,PS4_S,PM2_P)	Personal communication
21	7	c.1245+1G>T	p.?	Splice	Somatic	SCCOHT	Pathogenic(PVS1,PS4_P,PM2_P)	Registry
22	8	c.1408C>T	p.Gln470Ter	Nonsense	Germline	SCCOHT	Pathogenic(PVS1,PS4_P,PM2_P)	Testing lab
23	10	c.1648del	p.Leu550Trpfs*63	Frameshift	Germline	MRT	Pathogenic(PVS1,PS4_P,PM2_P)	Personal communication
24	10	c.1649del	p.Leu550Argfs*63	Frameshift	Germline	SCCOHT	Likely Pathogenic(PVS1,PS4_P)	Personal communication
25	10	c.1717del	p.Ala573Leufs*40	Frameshift	Germline	SCCOHT	Pathogenic(PVS1,PS4_P,PM2_P)	Poster presentation
26	10	c.1723C>T	p.Gln575Ter	Nonsense	Germline	SCCOHT	Pathogenic(PVS1,PS4_P,PM2_P)	Registry
27	10	c.1754_1757del	p.Lys585Argfs*27	Frameshift	Germline	SCCOHT	Pathogenic(PVS1,PS4_P,PM2_P)	Testing lab
28	10	c.1761+1G>A	p.?	Splice	Somatic	SCCOHT	Likely Pathogenic(PVS1_M,PS4_S,PM2_P)	Registry
29	12	c.1843del	p.Leu615Serfs*3	Frameshift	Unknown	SCCOHT	Pathogenic(PVS1,PS4_P,PM2_P)	Personal communication
30	12	c.1892dup	p.Asp632Argfs*19	Frameshift	Germline	SCCOHT	Pathogenic(PVS1,PS4_P,PM2_P)	Registry
31	12	c.1894del	p.Asp632Metfs*14	Frameshift	Germline	SCCOHT	Pathogenic(PVS1,PS4_P,PM2_P)	Poster presentation
32	12	c.1921dup	p.Ala641Glyfs*10	Frameshift	Germline	SCCOHT	Pathogenic(PVS1,PS4_P,PM2_P)	Registry
33	13	c.1985C>G	p.Ser662Ter	Nonsense	Somatic	SCCOHT	Pathogenic(PVS1,PS4_P,PM2_P)	Personal communication
34	13	c.1996G>T	p.Glu666Ter	Nonsense	Germline	SCCOHT	Pathogenic(PVS1,PS4_P,PM2_P)	Registry
35	14	c.(2001+1_2002-1)_(2123+1_2124-1)del	Exon 14 Deletion	Frameshift	Germline	SCCOHT	Pathogenic(PVS1,PS4_P,PM2_P)	Personal communication

36	15	c.2164C>T	p.Gln722Ter	Nonsense	Germline	SCCOHT	Pathogenic(PVS1,PS4_P,PM2_P)	Testing lab
37	15	c.2274+1G>A	p.?	Splice	Unknown	SCCOHT	Pathogenic(PVS1,PS4_P,PM2_P)	Personal communication
38	16	c.2287G>T	p.Glu763Ter	Nonsense	Germline	SCCOHT	Pathogenic(PVS1,PS4_P,PM2_P)	Testing lab
39	16	c.2311_2316del	p.Asn771_Asn772del	In-frame deletion	Germline	SCCOHT	VUS(PS4_P,PM2_P)	Personal communication
40	16	c.2406del	p.Asn803Metfs*28	Frameshift	Somatic	SCCOHT	Pathogenic(PVS1,PS4_P,PM2_P)	Registry
41, 42, 43	16	c.2438+1G>A	p.?	Splice	Somatic (x2), Germline (x1)	SCCOHT (x3)	Pathogenic(PVS1,PS4_S,PM2_P)	Registry
44	16-17	c.(2123+1_2124-1)_(2438+1_2439-1)del	Exon 16-17 Deletion	In-frame deletion	Germline	SCCOHT	VUS(PS4_P,PM2_P)	Testing lab
45	17	c.2453G>A	p.Trp818Ter	Nonsense	Somatic	SCCOHT	Pathogenic(PVS1,PS4_P,PM2_P)	Registry
46	17	c.2502C>A	p.Tyr834Ter	Nonsense	Germline	SCCOHT	Likely Pathogenic(PVS1,PS4_P)	Poster presentation
47	17	c.2506-2A>G	p.?	Splice	Unknown	SCCOHT	Pathogenic(PVS1,PS4_M,PM2_P)	Personal communication
48	18	c.2527del	p.Ala843Profs*15	Frameshift	Germline	SCCOHT	Pathogenic(PVS1,PS4_P,PM2_P)	Registry
49	19	c.2780_2783del	p.Phe927Cysfs*22	Frameshift	Germline	SCCOHT	Pathogenic(PVS1,PS4_P,PM2_P)	https://www.sciencedirect.com/science/article/pii/S221026121930687X
50	19	c.2838del	p.Phe947Leufs*3	Frameshift	Germline	SCCOHT	Pathogenic(PVS1,PS4_P,PM2_P)	Poster presentation
51	19	c.2854_2855delinsC	p.Glu952Glnfs*5	Frameshift	Germline	SCCOHT	Pathogenic(PVS1,PS4_P,PM2_P)	Poster presentation
52	19	c.2859+1G>A	p.?	Splice	Somatic	SCCOHT	Pathogenic(PVS1,PS4_S,PM2_P)	Registry
53	19	c.2860-1G>A	p.?	Splice	Germline	ATRT	Pathogenic(PVS1,PS4_P,PM2_P)	Testing lab
54	20	c.2935C>T	p.Arg979Ter	Nonsense	Germline	Non-specific	Pathogenic(PVS1,PS4_S)	Poster presentation

						ovarian		
55	21	c.3013C>T	p.Arg1005Ter	Nonsense	Somatic	SCCOHT	Pathogenic(PVS1,PS4_S)	Registry
56	22	c.3104del	p.Leu1035Argfs*2	Frameshift	Somatic	SCCOHT	Pathogenic(PVS1, PS4_P,PM2_P)	Registry
57	22	c.3168+2T>C	p.?	Splice	Germline	SCCOHT	Pathogenic(PVS1,PS4_M,PM2_P)	Poster presentation
58	24	c.3228C>A	p.Tyr1076Ter	Nonsense	Germline	SCCOHT	Pathogenic(PVS1,PS4_P, PM2_P)	Poster presentation
59, 60	24	c.3229C>T	p.Arg1077Ter	Nonsense	Germline (x2)	SCCOHT (x2)	Pathogenic(PVS1,PS4_S)	Registry
61	24	c.3277C>T	p.Arg1093Ter	Nonsense	Unknown	SCCOHT	Pathogenic(PVS1,PS4_S)	Personal communication
62	25	c.3547-1G>A	p.?	Splice	Germline	SCCOHT	Pathogenic(PVS1,PS4_P, PM2_P)	Personal communication
63	26	c.3565C>T	p.Arg1189Ter	Nonsense	Unknown	SCCOHT	Pathogenic(PVS1,PS4_P, PM2_P)	Poster presentation
64	26	c.3610_3615delCTCTGC	p.Leu1204_Cys1205del	In-frame deletion	Germline	SCCOHT	VUS (PS4_P,PM2_P, PM4)	Testing lab
65	28	c.3883G>T	p.Glu1295Ter	Nonsense	Unknown	SCCOHT	Pathogenic(PVS1,PS4_P, PM2_P)	Personal communication
66,67	28	c.3951+1G>A	p.?	Splice	Germline (x2)	SCCOHT (x2)	Likely Pathogenic(PVS1_S,PS4_M,PM2_P)	Registry
68	29	c.3982del	p.Ala1328Profs*30	Frameshift	Somatic	SCCOHT	Pathogenic(PVS1,PS4_P, PM2_P)	Registry
69	29	c.3997_3998insGGAAG	p.Pro1335Glyfs*25	Frameshift	Somatic	SCCOHT	Pathogenic(PVS1,PS4_P, PM2_P)	Registry
70	29	c.4170+2T>G	p.?	Splice	Somatic	SCCOHT	Likely Pathogenic(PVS1_S,PS4_M,PM2_P)	Registry
71	33	c.4622del	p.Lys1541Argfs*2	Frameshift	Germline	SCCOHT	Pathogenic(PVS1,PS4_P, PM2_P)	Testing lab
72	34	c.4763del	p.Val1588Alafs*40	Frameshift	Germline	SCCOHT	Pathogenic(PVS1,PS4_P, PM2_P)	Testing lab
73	17-25	c.(2438+1_2439-1)_(3546+1_3547-1)del	Exon 17-25 Deletion	Frameshift	Germline	SCCOHT	Pathogenic(PVS1,PS4_P, PM2_P)	Testing lab

VUS, Variant of Uncertain Significance; SCCOHT, Small-cell carcinoma of the ovary, hypercalcemic type; ATRT, atypical/typical rhabdoid tumor; MRT, malignant rhabdoid tumorVariants are annotated on SMARCA4 transcript NM_001128849.3.
[1]variant was detected by array, and therefore the breakpoints are approximate.

2. Functional Assays

In my initial thesis submission, I was unable to complete the functional assays outlined in Aim 3, Section 3.3. However, these assays were successfully completed for the final thesis submission, and the results are discussed in this section of the appendix. Short- and long-term cell viability assays were conducted on SCCOHT-1 cells with reexpression of various *SMARCA4* variants. Additionally, protein expression assays were performed to analyze the expression levels of SMARCA4, Cyclin D1, IP3R3, and CD44.

The short-term cell viability assay results, as shown in **Figure S1**, indicate that the Lv (control) exhibits the highest viability, serving as the reference (100%). The variants associated with SCCOHT (S1, S2, S3) and ATRT (ATRT1, ATRT2) demonstrate the highest viability, similar to the empty vector (Lv). Notably, the ATPase dead variant, contrary to expectations, does not result in significant cell death. In contrast, the wild-type (WT), benign (B), and non-truncating variants associated with CSS (CSS1, CSS2) exhibit significantly reduced viability compared to the control.

These observations align with the results obtained using the earlier constructs. Notably, SCCOHT-related variants display greater cell viability compared to both other variants and the control. In contrast, the benign, wild-type, and CSS-related variants show increased cell mortality, consistent with previous findings. Furthermore, the ATPase-dead variant exhibits reduced cell viability, though still higher than the controls, which also supports earlier results.

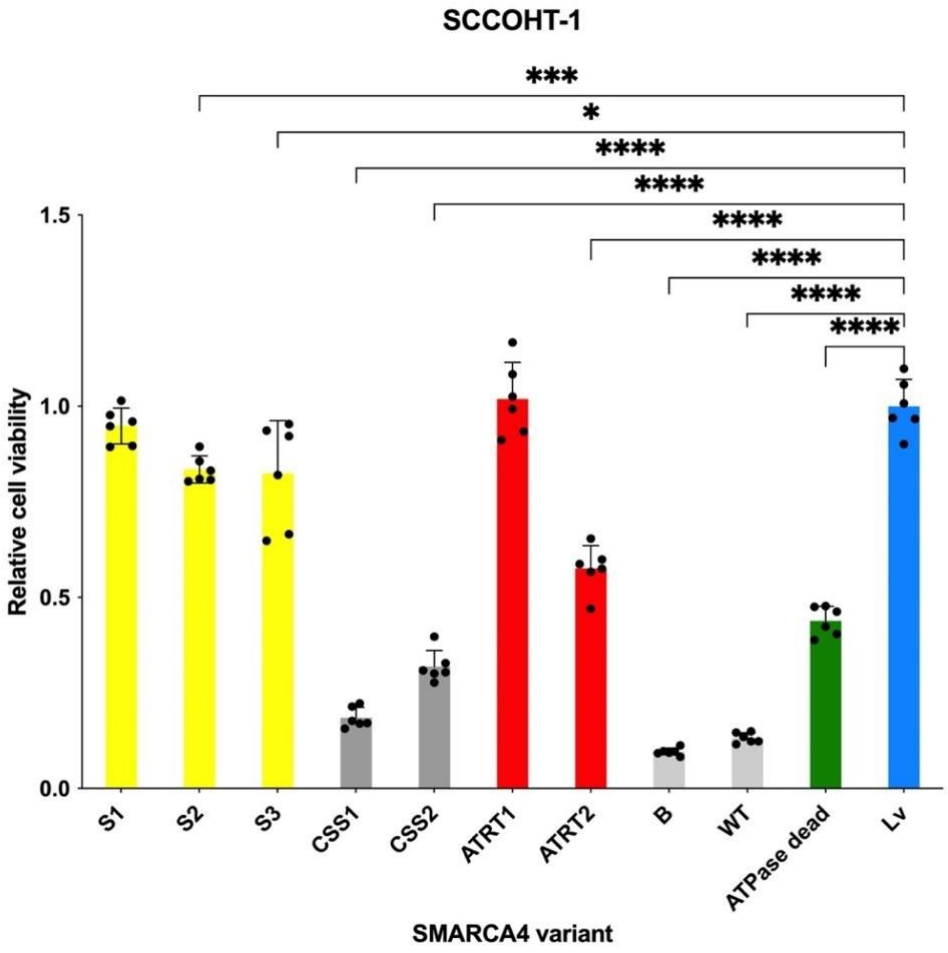

Figure S1. Short-term cell viability assay in SCCOHT-1 cells. The bar graph shows the cell viability percentages for different variants introduced: Lv (Control), WT (Wild Type), B (Benign variant), CSS1 (non-truncating variant causing CSS), CSS2 (non-truncating variant causing CSS), ATPase dead (ATPase dead variant), S1 (non-truncating variant causing SCCOHT), S2 (non-truncating variant causing SCCOHT), S3 (non-truncating variant causing SCCOHT), and ATRT1 (non-truncating variant causing ATRT) and ATRT2 (non-truncating variant ATRT). Error bars: mean ± standard deviation (s.d.) of replicates (n = 6; unpaired two-tailed t test, ****$p < 0.0001$, ***$p < 0.001$, *$p < 0.05$).

In alignment with the short-term cell viability assay, the long-term assay reveals that CSS-related and benign variants (CSS1, CSS2, B, WT) significantly inhibit SCCOHT-1 cell growth, resulting in cell death within 12 days (**Figure S2**). Notably, SCCOHT- and ATRT-related non-truncating variants (S1, S2, S3, ATRT1, and ATRT2) demonstrate minimal suppression, comparable to the empty vector (Lv, control). Interestingly, these variants exhibit even less suppression than the empty vector, which is an unexpected outcome. This discrepancy may be attributed to the fact that Lv cells were not allowed to recover adequately following antibiotic selection, potentially impacting long-term growth. The ATPase-dead variant also resulted in reduced cell survival, similar to the CSS-related variants.

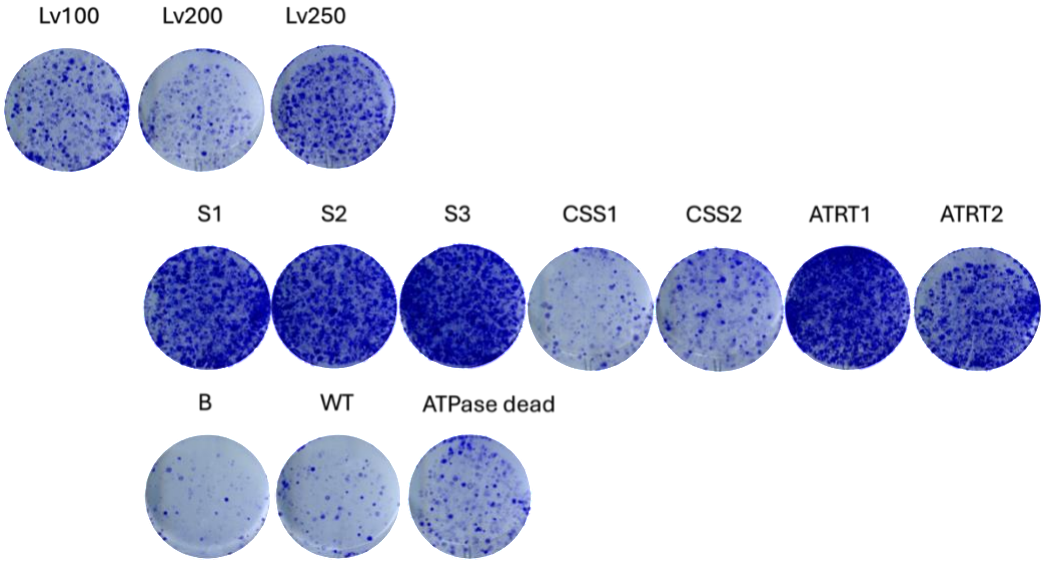

Figure S2. Long-term cell viability assay in SCCOHT-1 cells. Colony-formation assay of SCCOHT-1 cells expressing *SMARCA4* variants or control after 12 days of culturing. All dishes were fixed at the same time, stained, and photographed.

Furthermore, protein expression assays were conducted to examine the expression levels of SMARCA4, Cyclin D1, IP3R3, and CD44, with HSP90 and Beta-Actin serving as controls. As depicted in **Figure S3A**, SMARCA4 expression was observed across all variants, except for the empty vector (Lv), consistent with expectations. The loss of SMARCA4 in SCCOHT is

associated with Cyclin D1 deficiency. Notably, the reexpression of SCCOHT-associated missense variants in SCCOHT-1 cells failed to restore Cyclin D1 expression to levels comparable to those observed in CSS-related variants and controls (CSS1, CSS2, B, WT, and ATP) (**Figure S3A**). Similarly, IP3R3 expression was not restored in SCCOHT-associated missense variants, whereas CSS-related variants, ATRT1, B, WT, and ATP variants demonstrated restoration (**Figure S3B**). CD44 expression was detected in all variants (**Figure S3B**).

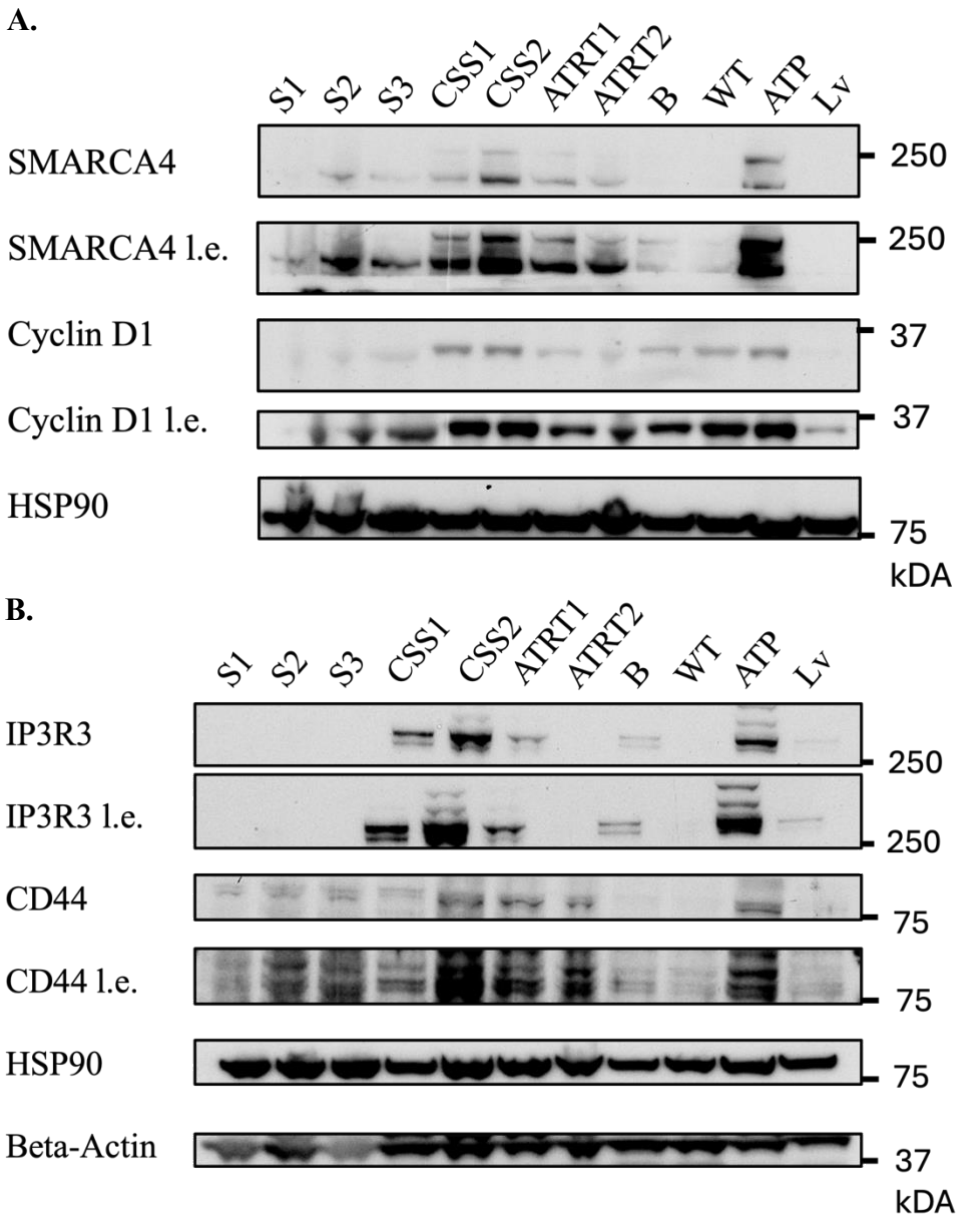

Figure S3. Western blot analysis for the indicated proteins in SCCOHT-1 cells. l.e. long exposure.

Cyclin D1 deficiency in SCCOHT is closely linked to the loss of SMARCA4. Its loss leads to the downregulation of Cyclin D1 expression in these cells. The restoration of SMARCA4 in SCCOHT-1 cells results in the upregulation of Cyclin D1, as previously reported.[32] However, our results show that the re-expression of missense variants associated with SCCOHT fails to induce Cyclin D1 upregulation following SMARCA4 restoration, distinguishing them from CSS-related variants that successfully restore Cyclin D1 levels.

In terms of IP3R3 expression, it has been observed that SMARCA4/2 directly activates the expression of this gene. SMARCA4/2 loss leads to reduced IP3R3 expression, which contributes to chemotherapy resistance in ovarian and lung cancer cells.[33] When SMARCA4 is restored in these cells, there is an increase in IP3R3 expression, which in turn promotes apoptosis, particularly in response to chemotherapy.[33] Notably, SCCOHT-associated missense variants do not restore IP3R3 expression upon SMARCA4 restoration, in contrast to CSS-related variants that do, paralleling the observations with Cyclin D1. This differential response provides a potential molecular basis for distinguishing missense variants that lead to SCCOHT from those associated with CSS.

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