2021

Mosaicism in tumor suppressor gene syndromes: prevalence, diagnostic strategies, and transmission risk

BOSTON UNIVERSITY

SCHOOL OF MEDICINE

Thesis

MOSAICISM IN TUMOR SUPPRESSOR GENE SYNDROMES: PREVALENCE, DIAGNOSTIC STRATEGIES, AND TRANSMISSION RISK

by

JILLIAN LEIGH CHEN

B.A., University of California, Berkeley, 2019

Submitted in partial fulfillment of the

requirements for the degree of

Master of Science

2021

Approved by

First Reader

Vickery Trinkaus-Randall, Ph.D.
Professor of Biochemistry

Second Reader

David Kwiatkowski, M.D., Ph.D.
Professor of Medicine
Harvard University School of Medicine

Third Reader

Krinio Giannikou, Ph.D.
Instructor of Medicine
Harvard University School of Medicine

DEDICATION

I dedicate this work to my family.

ACKNOWLEDGMENTS

I am grateful for the incredible mentorship of both Dr. Krinio Giannikou, my primary mentor, and Dr. David Kwiatkowski. With their guidance and dedication to teaching, I have learned an immense amount about the fields of cancer genetics, academic medicine, and scientific writing. The Kwiatkowski Lab's work on Tuberous Sclerosis Complex and mosaicism serves as a foundation for this literature review and model for other tumor suppressor gene syndromes.

MOSAICISM IN TUMOR SUPPRESSOR GENE SYNDROMES: PREVALENCE, DIAGNOSTIC STRATEGIES, AND TRANSMISSION RISK

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ABSTRACT

Mosaicism occurs due to postzygotic genetic alterations during early embryonic development. The phenomenon is common, present in all humans, animals, and plants, and is associated with phenotypic variability and heterogeneity. Mosaic pathogenic gene variants result in a mosaic disease state, in which the individual can present with mild, generalized disease, a localized disease phenotype in specific organs and tissue regions, or full-blown clinical features which are indistinguishable from the heterozygous disease state. Multiple studies have described the prevalence and clinical correlations associated with low-level mosaicism for various genetic disorders, including several tumor suppressor gene (TSG) syndromes, which are well-known to display mosaicism. However, the extent of mosaicism research varies widely between TSG syndromes. Currently there is no comprehensive, up to date review covering multiple TSGs and focusing on mosaicism prevalence, diagnostic strategies and transmission risk.

Here, in this literature review, I focus on 8 common tumor suppressor genes *NF1, NF2, TSC1, TSC2, RB1, PTEN, VHL,* and *TP53*; reporting the following disease aspects:

- Role and function of each tumor suppressor gene, disease prevalence, inheritance pattern, penetrance/expressivity pattern, age of onset clinical features, organs affected, and benign or malignant tumors seen
- Different types of mosaicism, including critical review of recent, representative publications for each tumor suppressor gene syndrome

- Established criteria for clinical diagnosis of inherited versus mosaic disease, molecular diagnosis, and current methods of genetic analysis

Then more extensively, this thesis discusses the most informative, representative original studies for each TSG and provides a summary which covers:

- The number of mosaic patients analyzed and the spectrum of clinical features of the cohort they were sampled from
- The spectrum of variant allele frequency (VAF), tissue types analyzed, and different analysis methods performed
- Whether or not the mosaic patients met clinical criteria for diagnosis of inherited disease
- The number of patients who were persistently classified as no mutation identified (NMI) after genetic analysis
- Spectrum and type of mosaic mutational event(s) identified
- Age of onset and age range of mosaic patients
- Patient ascertainment and family history (sporadic or familial cases) and
- Type of mosaicism seen

Furthermore, it compares and discusses disease severity, possibility of malignancy, and genotype-phenotype correlations for each TSG. Ultimately, by juxtaposing these TSGs, this review aims to centralize existing knowledge about mosaicism and provide insight into how molecular techniques can be broadly applied for better diagnosis of mosaic disease.

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ACC	Adrenocortical Carcinoma
AF	Angiofibroma
BRRS	Bannayan Riley-Ruvalcaba Syndrome
BU	Boston University
CHIP	Clonal Hematopoiesis of Indeterminate Potential
CNLOH	Copy-Neutral Loss of Heterozygosity
COLD-PCR	CO-amplification at Lower Denaturation temperature-PCR
CPC	Choroid Plexus Carcinoma
CS	Cowden Syndrome
CSGE	Conformational Sensitive Gel Electrophoresis
DGGE	Denaturing Gradient Gel Electrophoresis
DHPLC	Denaturing High-Performance Liquid Chromatography
ERK	Extracellular Signal-regulated Kinase
FAK	Focal Adhesion Kinase
FISH	Fluorescence *In Situ* Hybridization
GFR	Growth Factor Receptor
GRB2	Growth Factor Receptor-Bound protein 2
HIF1α	Hypoxia Inducible Factor-1α
ID	Intellectual Disability
LFS	Li-Fraumeni Syndrome
LOH	Loss of Heterozygosity
MAPK	Mitogen-Activated Protein Kinase

MCF	Mucocutaneous Findings
MPS	Massively Parallel Sequencing
mTORC1	mammalian Target of Rapamycin Complex 1
NIH	National Institutes of Health
NF1	Type 1 neurofibromatosis
NF2	Type 2 neurofibromatosis
NGS	Next-Generation Sequencing
NMI	No Mutation Identified
PCR-SSCP	Polymerase Chain Reaction-Single-Strand Conformation Polymorphism
PGD	Preimplantation Genetic Diagnosis
PI3K	Phosphoinositide-3-Kinase
PIP3	Phosphatidylinositol 3,4,5-triphosphate
PS	Proteus Syndrome
PV	Pathogenic Variant
QMPSF	Quantitative Multiplex PCR of Short Fluorescent Fragments
Rb	Retinoblastoma
RCC	Renal Cell Carcinoma
RHEB	Ras-Homolog Enriched in the Brain
RTK	Receptor Tyrosine Kinase
SEGA	Subependymal Giant-cell Astrocytoma
SEN	Subependymal Nodule
SOS	Son of Sevenless

SSCP	single-strand conformation polymorphism
TGGE	Temperature Gradient Gel Electrophoresis
TSC	Tuberous Sclerosis Complex
TSG	Tumor Suppressor Gene
TSS	Tumor Suppressor gene Syndrome
VAF	Variant Allele Frequency
VHL	Von Hippel-Lindau
VS	Vestibular Schwannoma
WES	Whole Exome Sequencing

INTRODUCTION

Background on Mosaicism

Mosaicism is a phenomenon characterized by the presence of two or more genetically distinct cell populations in a human developed from a single zygote. Once fertilization occurs, a developing zygote is subject to spontaneous genetic alterations, a subset of which escape correction by the inherent DNA correction machinery in the cell. These spontaneous mutations can give rise to multiple cell lineages with distinct genomes all derived from a single egg, resulting in a mosaic individual[1] that may demonstrate cell and tissue heterogeneity and phenotypic variability. The phenomenon is common and present in all humans, but the vast majority of these alterations affect the non-coding genome and may or may not have an apparent effect. However, spontaneous genetic alterations can be pathogenic and give rise to genetic disorders, thus causing a *de novo* case of hereditary disease. Mosaicism is characterized by the presence of a genetic variant at variant allele frequency (VAF) <50% in one or more tissues and/or cell types.

Mosaicism is distinct from both chimerism and X-chromosome inactivation (XCI), which cause phenotypic heterogeneity in individuals by different mechanisms. In chimerism, separate fertilized eggs fuse to form an embryo.[2] In XCI, first observed by Mary Lyon in 1961, random epigenetic silencing causes expression of only 50% of an individual's X chromosomes and does not change cells' genomic content.[3] The cell and tissue types that are affected by a mosaic mutation are variable and unpredictable. Low-allele frequency mosaic mutations may cause clinicopathological features, and are generally impossible to distinguish from somatic events in a single cell clone in an individual. The proportion of mosaic mutant cells can be as low as 0.02%.[4]

It was not always clear that mosaicism was a common phenomenon. First discussed in the early 20th century when plants and animals were observed to display a "mosaic-like distribution" of phenotypic traits -- colorless spots on seeds of maize[5] and tortoiseshell fur on guinea pigs, for instance,[6] no knowledge of genes or mutations yet existed to explain these occurrences of mosaic coloring. Phenotypic features were similarly seen in humans -- patterned skin discoloration, hyperkeratosis, atrophy, and hair distribution all gave rise to early hypotheses about mosaicism.[2] In 1895, Alfred Blaschko, a German dermatologist had already proposed that a "disturbing factor" during differentiation of a developing zygote was responsible for the isolated appearance of linear epidermal nevi, now termed the lines of Blaschko. In other words, the timing of the postzygotic disturbance ensured that only certain patches of skin were affected while other regions remained normal.[7]

The development of advanced molecular techniques and sequencing of the whole genome not only expanded the range of mutation types detected but also brought to light that mosaicism can affect all cell types beyond what is initially observable on the skin. As Blaschko theorized, it is now known that the extent of mosaic disease depends on when during postzygotic development the mutation happens. Mosaicism is categorized as *gonadal/germline* when the mutation affects the gametes or as *somatic* if only body cells contain the mutation. Additionally, *generalized* or *localized* (**Figure 1**) mosaicism refers to the body distribution and extent of affected tissues.

In generalized mosaicism, the alteration is acquired very early in embryogenesis during the first few cell divisions and before differentiation.[8] This early mutation means that cells bearing the genetic variant will give rise to all cell and tissue types. However, although disseminated in this manner, such individuals will often have a milder phenotype than the

inherited full heterozygous form of disease.[9] When a mutation is acquired at a later stage of postzygotic development, after differentiation has occurred, the disease phenotype may be localized to certain organs and/or tissue types. If only the testes in males or ovaries in females are affected, the individual is considered to have gonadal or germline mosaicism. Germline mosaic variants can be inherited and transmitted to the individual's offspring. Also, if an individual contains mosaic pathogenic variants in both somatic and germinal cells, they are classified as *gonadosomatic*.[8] These classifications are better considered as points on a spectrum rather than as discrete categories, as it is unlikely that a mosaic mutation would be completely confined to either somatic or germ cells.[2] It is common for the variant allele frequency (VAF) to differ among the affected tissues and organs. This reflects the chance distribution of cells carrying *de novo* mosaic variants among the progenitor cells for different tissues during embryogenesis.

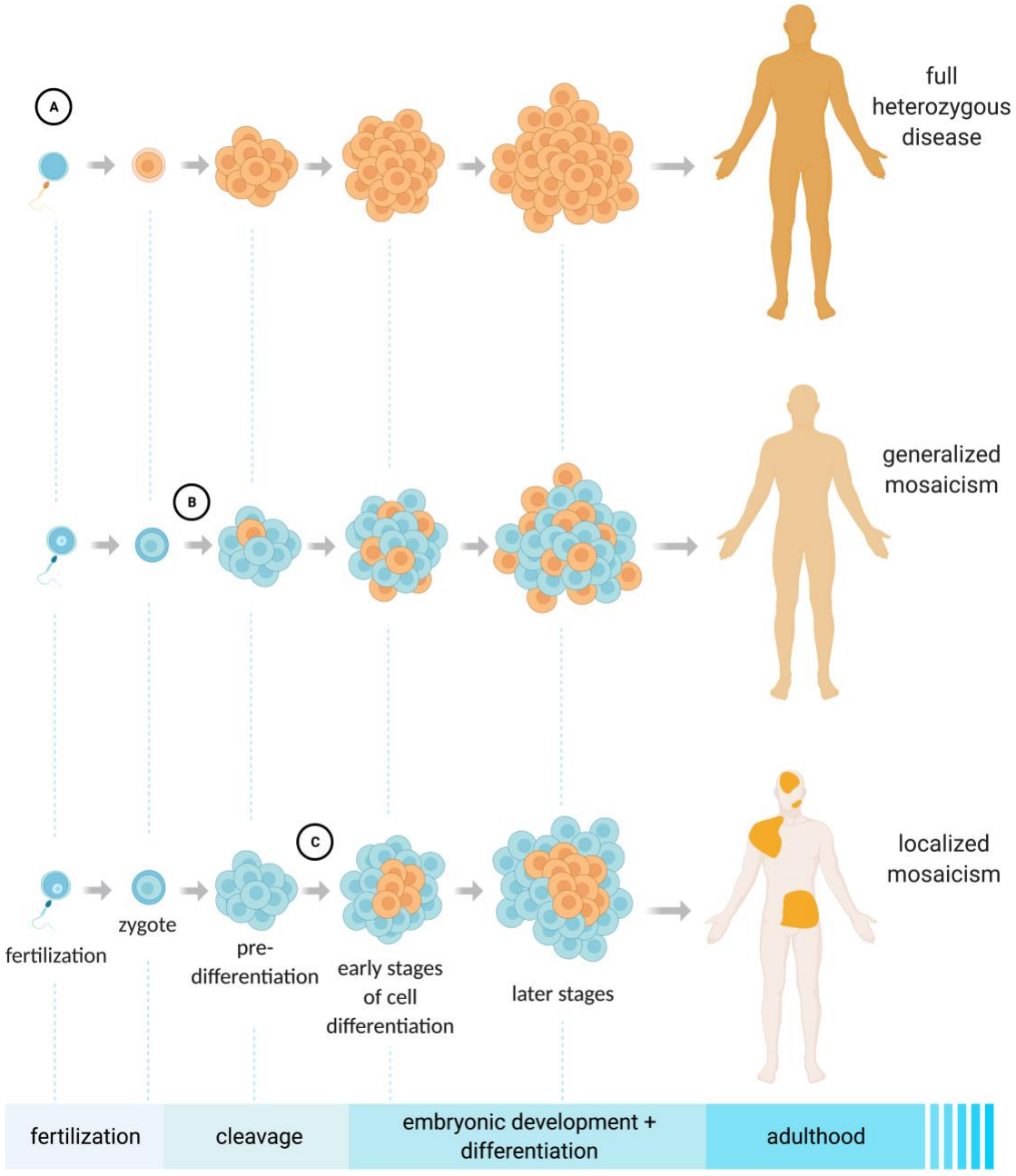

Figure 1 | Mosaicism in human disease. The extent of mosaicism depends on timing and location of the genetic alteration. Mosaicism can be either germline (seen in gametes) or somatic (present in body cells only). **a.** A germline genetic event occurs in sperm and/or egg. The resulting zygote is heterozygous for the alteration, which is carried into all three germ layers. The fully-grown offspring has full heterozygous disease. **b.** A somatic alteration

acquired in the blastocyst stage, prior to the zygote's differentiation into the germ layers of the future embryo, will usually lead to presence of the mutation in multiple and/or all cell types. **c.** A somatic mutation acquired at a later stage in embryogenesis will be found only in the progeny cells derived from that single cell. As a genetic event occurs later and later in development, fewer tissues will be affected by the alteration. Ultimately, only cells within the affected lineages and tissue types have the genetic alterations, resulting in localized mosaicism.

Tumor Suppressor Genes and Associated Pathways

Tumor suppressor genes (TSGs) are genes that play a role in regulating normal cell growth and proliferation, and thus their loss from a cell can lead to tumor growth. They function in an opposite manner from oncogenes, which promote tumor development through functional gain. Whereas gain-of-function mutations are cancer-causing in oncogenes, loss-of function mutations in TSGs contribute to tumorigenesis, as well.[10] Multiple TSGs are involved in the central growth regulatory pathways active in all cells, the *PI3K*/*AKT*/*mTOR* and Ras/MEK/ERK pathways (**Figure 2**), and result in tumor-predisposition and tissue overgrowth syndromes when mutated.[11] Related syndromes are classified by the presence of specific and unique clinical features.

mTOR, a serine-threonine kinase, can form one of two complexes: mTOR complex 1 (mTORC1) or mTOR complex 2 (mTORC2), each of which gives way to distinct downstream events that promote cell survival, protein synthesis, and proliferation. GTP-bound Ras-homolog enriched in the brain (RHEB) activates mammalian target of rapamycin complex 1 (mTORC1), a central regulator of cell growth and proliferation, and lies downstream of the PI3K pathway.[9] The active TSC protein complex hydrolyzes GTP bound to RHEB, suppressing mTORC1 activity.[9] Thus, input from the PI3K and Ras pathways activates mTORC1 through inhibition of the TSC protein complex. When core components

of the TSC protein complex, such as TSC2 are completely lost from a cell, dysregulation and constitutive activation of mTORC1 can lead to uncontrolled cell proliferation.[11]

While the exact pathway upstream of mammalian target of rapamycin complex 2 (mTORC2) is not well defined, mTORC2 is known to be required for full, enhanced AKT activation, and indirectly contributes to mTORC1 activity by removing TSC-mediated inhibition of RHEB.[9,12] In low energy states, high AMP levels activate AMPK, which stimulates TSC2 and inhibits mTORC1 activity.[9]

Like *TSC2*, other tumor suppressor genes (TSGs) act within the two ubiquitous pathways and regulate cell growth, proliferation, and metabolism. Specifically, *NF1* encodes a GTPase-activating protein, neurofibromin, which regulates the RAS-cAMP pathway and mitogen-activated protein kinase (MAPK) pathway.[13] *NF2* is also known to act within the PI3K pathway as evidenced by elevated levels of phosphorylated AKT in NF2 tumor tissue in one study.[14] Additionally, elevated phospho-ERK and phospho-MEK have been measured in NF2 tumors. *PTEN* is a phosphatase that regulates mTORC1 by dephosphorylating PIP3.[15] *NF2* and *PTEN* have additional functions described later. *VHL*, *TP53,* and *RB1* products have tumor suppressor functions outside of the two pathways discussed here. *VHL* plays a role in regulating the hypoxia-inducible factor complex, maintaining normal levels of blood vessel formation. *TP53* acts within the nucleus to regulate repair mechanisms and apoptosis following DNA damage. *RB1* also influences cell survival and apoptosis through interactions with other nuclear proteins (VarSome: The Human Genomic Variant Search Engine, Genes: *VHL, TP53, RB1* respectively).[16]

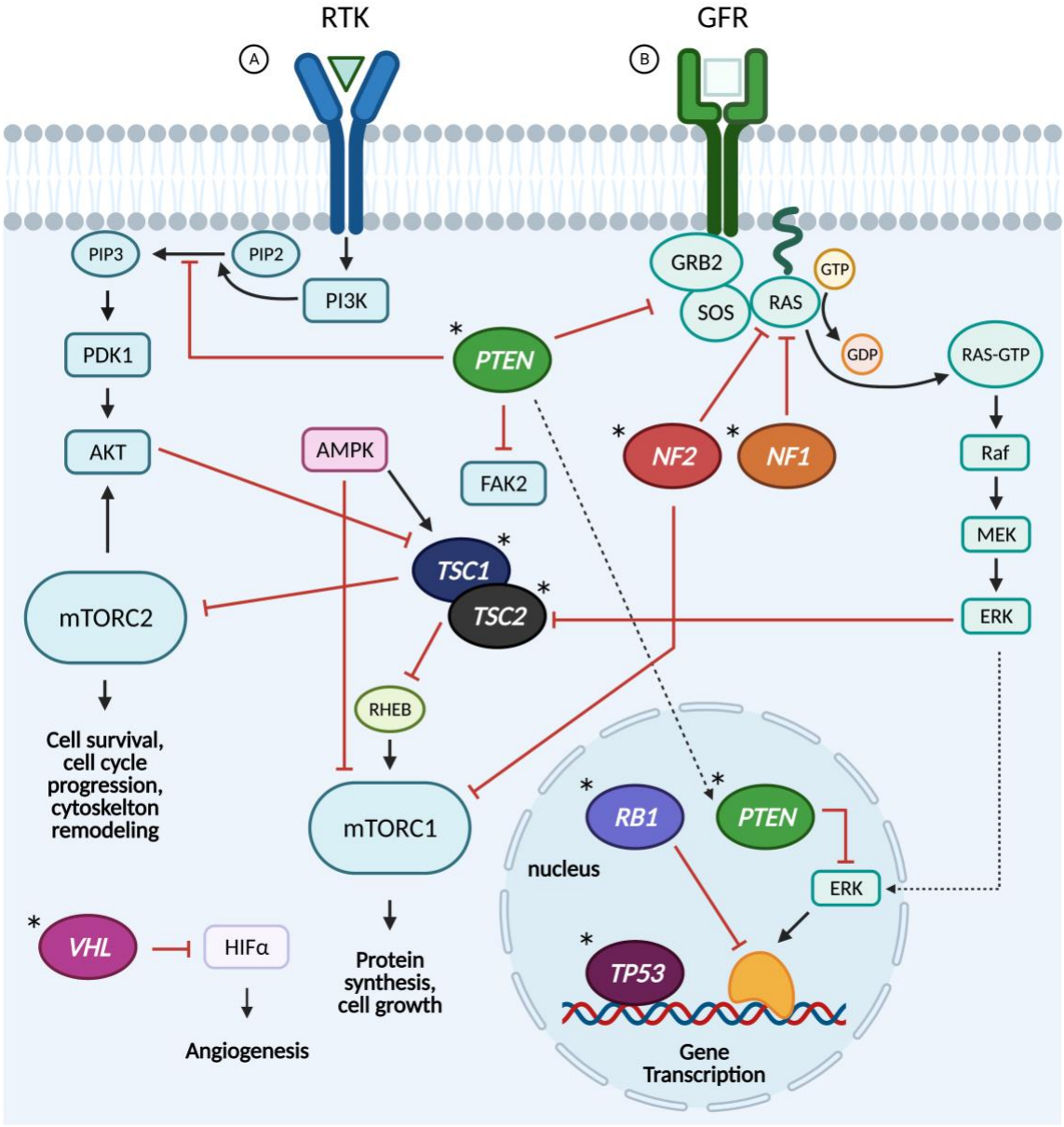

Figure 2 | Tumor suppressor genes involved in the *PI3K/AKT/mTOR* and Ras pathways. Amino acid levels, growth ligands (e.g. EGFR), and insulin provide information about the energetic and nutritional status of a cell, signaling through the (**A**) PI3K/AKT/mTOR and (**B**) Ras pathways to control cell growth and metabolism. Growth factors and nutrients stimulate growth factor receptors (GFR), also known as receptor tyrosine kinases (RTK), activating growth factor receptor-bound protein 2 (GRB2) and Son of Sevenless (SOS), a guanine nucleotide exchange factor. Following exchange of GDP for GTP, GTP-bound Ras initiates a phosphorylation cascade, ultimately activating extracellular signal-regulated kinase (ERK).[12] Growth factors, such as insulin, also stimulate the

phosphoinositide-3-kinase (PI3K) pathway. Activation of the insulin receptor tyrosine kinase (RTK), activates PI3K, which phosphorylates PIP2 to become PIP3. PDK1 is recruited to the membrane and allows activation of AKT.[17] When activated, both ERK and AKT phosphorylate and inhibit TSC2, a tumor suppressor gene product.[9,12] *PTEN*'s phosphatase activity indirectly inhibits the action of PI3K by dephosphorylating PIP3 back to PIP2. Tumor suppressor genes are marked with a star.

Individuals who inherit a heterozygous TSG mutation will develop a tumor suppressor gene syndrome (TSS) that predisposes the subject to tumorigenesis.[18] All known TSSs follow an autosomal dominant pattern of inheritance.[11] The Knudson two-hit model hypothesis has been confirmed as the primary mechanism of tumor development in TSS. Following Knudson's model, tumorigenesis results from deletion or inactivation of both functional TSG alleles.[18] For example, retinoblastoma development relies on biallelic inactivation of the *RB1* gene. The "first hit" *RB1* mutation may be inherited or sporadically acquired after birth. A sporadic "second hit" is necessary for the retinoblastoma to form. In the case of a sporadic first hit, tumor onset is at a later age.[19]

There are some exceptions to Knudson's hypothesis. For certain clinical features of some TSSs, biallelic inactivation of the TSG does not does not guarantee tumor development. This is exemplified by the malignant peripheral nerve sheath tumor's reliance on *TP53* inactivation in addition to biallelic *NF1* mutations in type 1 neurofibromatosis.[20] Additionally, haploinsufficiency, or presence of one mutant allele, either inherited or somatically acquired, is sufficient to cause formation of lesions or tumors. This has been demonstrated in the syndromes of *NF1, PTEN,* and *TP53.* [19] Some tumors in patients with Li-Fraumeni syndrome, caused by germline *TP53* mutations, have been demonstrated to contain only single-"hits" inactivating one *TP53* allele. Haploinsufficiency of the *PTEN* gene has been demonstrated to account for benign polyps and developmental disorders in *PTEN*

hamartoma tumor syndrome (PHTS).[21] *PTEN* demonstrates haploinsufficiency in 70-80% of prostate cancer cases and in 30-40% of breast cancer cases, while biallelic loss of *PTEN* is only seen in 5% of breast cancer patients.[21] One- and two-hit tumorigenesis in TSS arises by several different mechanisms as described below. (**Figure 3**).

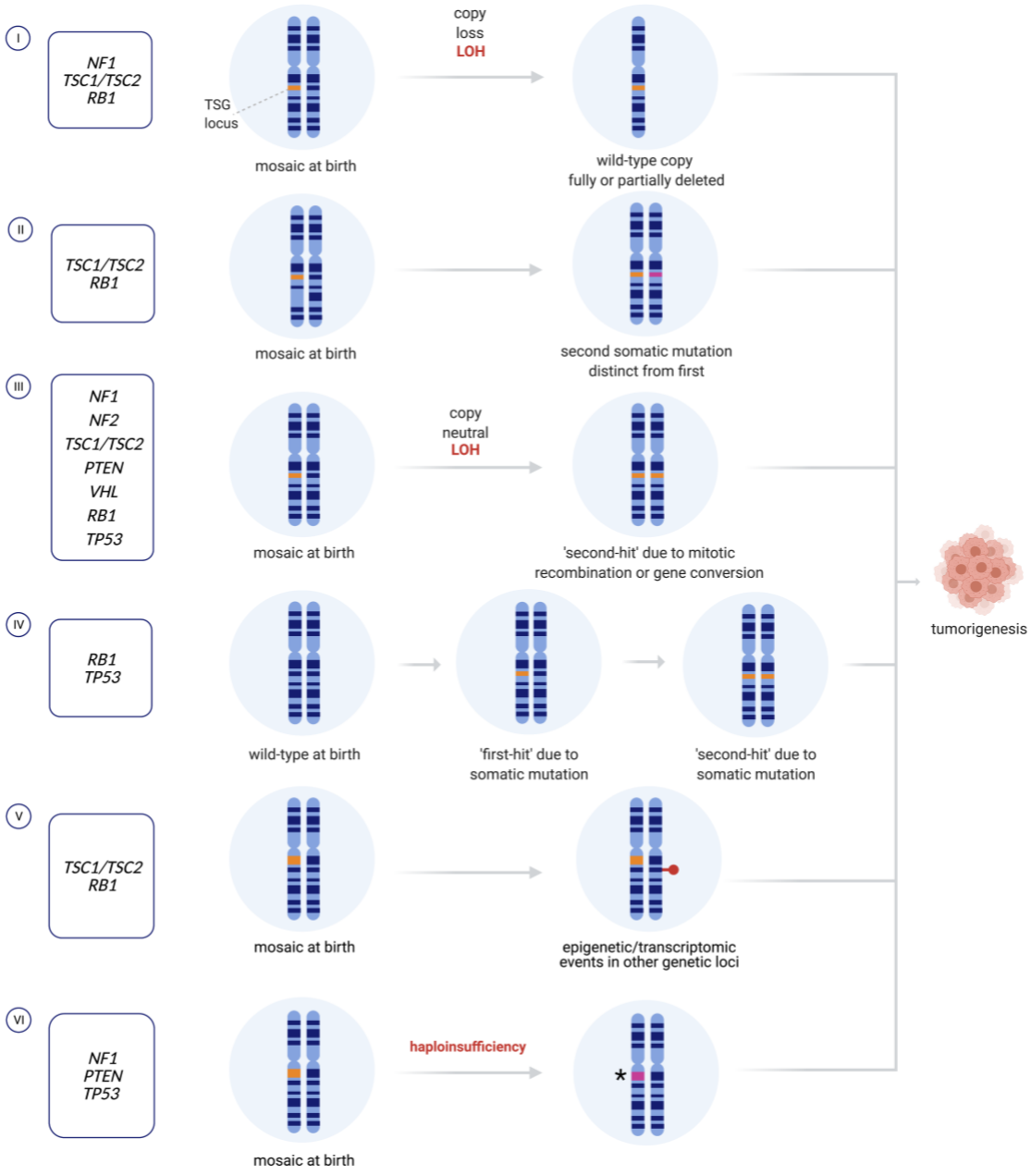

Figure 3 | Knudson's two-hit hypothesis and mechanisms in TSG mosaicism. Several mechanisms of two-hit loss occur for TSGs leading to tumor development. The first three mechanisms (I-III) apply to heterozygous in addition to mosaic individuals. **I)** An individual is somatic or germline mosaic at birth for a TSG mutation. Copy loss LOH (CL-LOH) occurs when all or part of the second functional allele is deleted. **II)** An individual acquires a second inactivating mutation that is distinct from the inherited germline one. **III)** Copy neutral LOH (CN-LOH) occurs due to a random mitotic homologous recombination event

or when the mutant allele is duplicated before or after the LOH event.[22,23,24] **IV)** Rarely, an individual who is wild-type at birth may acquire both "hits" sporadically. **V)** Currently, epigenetic and transcriptomic events are being investigated as potential contributors to tumorigenesis.[25] **VI)** Haploinsufficiency occurs in TSGs when a single-copy genetic event is capable of producing aberrant TSG function and causing tumor/lesion formation.[22]

Further studies are required to understand the specific role of LOH in each TSS. Interestingly, every TSG has its own distinctive set of tumors to which it predisposes. The reasons for this are not completely known and understood. Mosaic mutations in TSGs typically result in an attenuated or milder syndrome than what is seen in individuals with a full heterozygous mutation. This milder syndrome may include a later age of onset, or lack of some common diagnostic features with retention of others for the particular TSG syndrome. In this review, I will describe mosaicism seen in syndromes related to eight tumor suppressor genes: *NF1, NF2, TSC1, TSC2, RB1, PTEN, TP53,* and *VHL*. I will use the following classification system: high level mosaicism (VAF 20-35%), moderate level mosaicism (5-20%), low level mosaicism (VAF 1-5%), and very low level mosaicism (VAF <1%).[8]

Evolution of Molecular and Genetic Techniques

Historically, Sanger sequencing was one of the first technologies used for sequencing of the human and other genomes. Although revolutionary at the time, Sanger sequencing has limited sensitivity to identify mosaic mutations with a limit detection threshold of 10-15% VAF.[26] Moderate, low, and very low-level VAF mosaic cases are often masked or impossible to be identified using Sanger sequencing. Additionally, at higher levels of VAF, it can be difficult to distinguish mosaic from inherited germline mutations.[26] Sanger sequencing, multiple ligation dependent probe amplification (MLPA) for copy number alterations

(deletions, and gains/amplifications), and cytogenetic analyses such as Fluorescence In Situ Hybridization (FISH) are still in use for simple and thorough detection of higher frequency mutations and structural variants.[13,27]

The development of more sensitive and high throughput techniques, specifically massively parallel sequencing (MPS), has facilitated genetic analysis, enabling detection of very low-level VAFs (<5%) in mosaic patients. For some TSSs, MPS has improved sensitivity of detection for VAF levels down to 0.02%,[4] and is to date, the gold standard of screening for mosaic disease.[27] Apart from blood and saliva, which are the most common tissue samples analyzed, additional tissues and tumor samples may be analyzed when available, thus increasing the detection of low mosaicism. Mosaic mutations can be validated using different approaches, e.g. amplicon MPS and digital PCR, a high sensitivity method that allows assignment of somatic allele loss and estimation of tumor cell proportions.[20-28] Additionally polymorphism-based analyses, such as SNP mapping arrays,[29] have been used to detect loss of heterozygosity (LOH). However, these methods are not sufficiently sensitive to detect LOH in tumor samples that have low purity, reflecting a small fraction of tumor cells relative to normal cells.[28]

Literature Review Strategy

Mosaicism is well-studied in several tumor suppressor gene syndromes, e.g., Tuberous Sclerosis Complex (TSC), but poorly understood in others, such as *PTEN*-hamartoma tumor syndrome and Li-Fraumeni syndrome.[30,31] This paper aims to provide readers with a succinct overview of mosaicism in each of eight tumor suppressor gene syndromes, which are among

the most common and most well-studied and are implicated in the same canonical growth signaling pathways (**Figure 2**).

I reviewed literature on mosaicism extensively to best understand progress in the field. PubMed searches included key words for one of the following genes or syndromes, "NF1," "neurofibromatosis," "neurofibromatosis type 1," "NF2," 'neurofibromatosis type 2," "TSC," "tuberous sclerosis," "PTEN," "PTEN hamartoma tumor syndrome," "RB1," "retinoblastoma," "VHL," "Von-Hippel Lindau" "TP53," in conjunction with either "mosaic" or "mosaicism." Due to space limitations, I describe here the most relevant, original, and comprehensive studies focusing on large patient cohorts and representative case reports meeting diagnostic criteria for each syndrome from peer-reviewed, high quality journals. Small cohorts providing relevant information about mosaicism or using new sequencing technologies were also included. Case reports were included if they met standard diagnostic criteria for the disease or if they provided new considerable input about mosaicism.

I regret that I could not report all well-described studies and acknowledge the contribution of all scientists in each field. I aimed to avoid repeating already-reported information and focused on providing new input from the most current bibliography that has not been reported in a review format. By assessing newly established clinical and molecular diagnostic strategies that have facilitated detection of mosaicism in more well-studied syndromes, I hope to provide future directions and suggestions for better recognition of mosaicism in other less well studied TSS.

NF1 – Neurofibromatosis type 1

Type 1 neurofibromatosis (NF1) is caused by inactivating variants in *NF1*, on chromosome 17q11.2 (Online Mendelian Inheritance in Man, OMIM®. Johns Hopkins University, Baltimore, MD. MIM Number: {162200,613113}). NF1 affects approximately 1 in 3,000 births, regardless of sex or ethnicity.[20] The average age of symptom onset is at birth or in the first few years of life with 70% of patients presenting with one feature of NF1 by the first year of life, 97% developing two features by 8 years of age, and all patients displaying marked features by age 20.[32] The neurofibroma, a nerve sheath tumor associated with either spinal, peripheral, or cranial nerves, is the hallmark clinical feature of NF1.[20] Clinical diagnosis is met when a patient is found to have two of the following features: ≥6 café-au-lait spots, ≥2 neurofibromas or ≥1 plexiform neurofibroma, axillary or inguinal freckling, ≥2 Lisch nodules (iris hamartomas), optic nerve glioma, bony changes such as dysplasia of sphenoid bone, or thinning of long bone cortex that may be accompanied by pseudoarthritis, or a first-degree relative with NF1.[32] Malignancy is the most frequent cause of mortality – peripheral nerve sheath tumors, gliomas, leukemia, pheochromocytomas, gastrointestinal stromal tumors are the malignancies most commonly associated with NF1 (**Figure 4**). Life expectancy in patients with NF1 is decreased by 8-21 years.[33,20]

A total of 6457 *NF1* variants[16] have been identified (VarSome database-updated March 30, 2021, Gene: *NF1*) in type 1 neurofibromatosis.[13] The most commonly identified mutations in NF1 are large deletions that encompass the gene and its flanking regions, also called microdeletions. These are identified in 4.7-11% of all NF1 patients.[34] Type I microdeletions encompass 1.4 Mb and comprise 70-80% of all microdeletions, while Type II

microdeletions encompass 1.2 Mb and comprise at least 10% of microdeletions.[35] Approximately 91% of type I microdeletions are maternally inherited, and 63% of type II microdeletions are mosaic.[35] Overall, microdeletions are associated with severe phenotypes[35] and a higher risk of developing MPNSTs (16-26%).[34] Mechanisms of lesion formation vary by clinical manifestation. Café-au-lait macules require biallelic inactivation while development of malignant nerve sheath tumors requires additional pathogenic variants, such as *TP53* mutations.[20] Some features also result from haploinsufficiency or modification by hormones.[20] Due to the *NF1* gene's large size, 61 exons (OMIM #613113), and wide variation in PVs, molecular testing requires blood analysis of genomic DNA and mRNA in addition to FISH to screen for large deletions in NF1. These methods combined are capable of detecting >95% of *NF1* mutations. However, wider tissue sampling, from café-au-lait macules or other skin lesions, is required for detection of mosaic cases.[20]

Around 50% of *NF1* mutations are *de novo*.[36] In contrast to the relatively common inherited NF1, mosaic NF1 is present in the general population at a rate of 1 in 36,000 to 40,000 individuals.[37] Both localized and generalized mosaicism have been identified in NF1, and mosaic cases present with consistently milder phenotypes and fewer clinical manifestations than inherited NF1. Typically, mosaic NF1 presents with classic café-au-lait spots, dense regions of melanocytes,[20] whose arrangement and symmetry depends on the timing of the initial mosaic mutation.[37]

Selumetinib, a MEK1 and MEK2 inhibitor in the MAPK pathway, is approved for the treatment of plexiform neurofibroma, and has shown 70% tumor reduction and a 68% improvement of related features.[38] Clinical trials conducted thus far have shown that MPNSTs are refractory to treatment.[20]

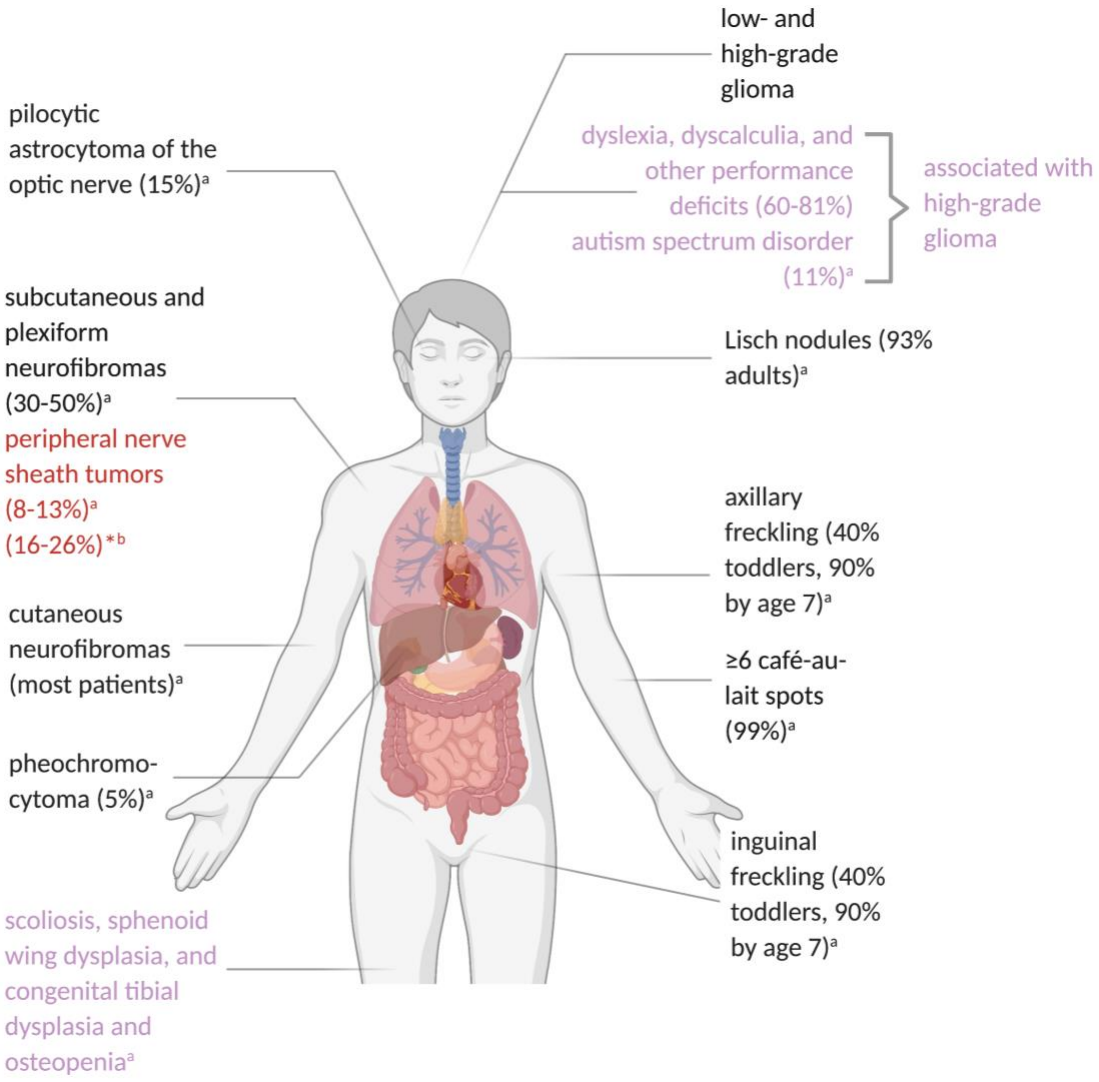

Figure 4 | Clinical features of Neurofibromatosis Type I. The peripheral nerve sheath tumor is the most frequent malignancy associated with NF1. Children with NF1 have a 7-fold elevated risk of hematopoietic malignancies, particularly myeloid leukemia, and an elevated risk of rhabdomyosarcoma, predominantly of the prostate gland and bladder. Women with NF1 under 50 years have an approximately 5-fold elevated risk of breast cancer. *Patients with *NF1* microdeletions have a higher risk of MPNST than those with intragenic deletions. Red text indicates malignancy. Black text indicates benign features. Symptoms associated with tumor growth are included in purple.
a. Farschtschi S et al., Dtsch Arztebl Int, 2020 b. Kehrer-Sawatzki H et al., Hum Genet, 2017

In a systematic review of mosaic patients reported between 1997 and September 2012, 157 individual case reports and two series of 124 and 39 patients were identified. Only 15 patients from the case studies had received genetic testing, 12 of whom had *NF1* variants.[37] Four of the mosaic patients had offspring with full NF1 and 11 developed malignancies, 3 of which were MPNSTs.[37] Because NF1 is usually diagnosed based on clinical features, molecular genetic testing was not frequently conducted in the past.[39] Since 2012, 258 additional mosaic patients have been reported in case reports and seven cohort studies (as of January 18, 2021).[13] Mosaicism is not well reported in NF1 compared to other autosomal dominant diseases.[40] and it is likely underdiagnosed. This may be because *NF1* deletion screening was previously limited to patients with overt features of NF1, including intellectual disability (ID) and facial dysmorphism.

Of note, Kehrer-Sawatzki et al. identified 8 mosaic patients out of 20 sporadic NF1 cases in 2004 using FISH. None of the patients analyzed had ID or facial dysmorphism. Interestingly, the peripheral blood samples of all mosaic patients contained 91-100% cells with deletions.[41] However, further tissue sampling of buccal mucosa, skin fibroblasts, or neurofibroma tissue revealed variant frequencies between 51-80%. Mosaic proportions were only seen in buccal cells and neurofibroma tissue. It was proposed that hematopoietic stem cells containing an *NF1* deletion have a growth advantage over normal cells. 7 of 8 mosaic patients demonstrated a Type II microdeletion, encompassing 13 genes.[41] While Type I microdeletions have breakpoints within *NF1* low-copy repeats, Type II microdeletion breakpoints occur in the *JJAZ1* gene (17q21 [OMIM, #606245]), which is highly expressed in the cerebellum, Purkinje fibers, and pyriform cortex.[41] *JJAZ1* haploinsufficiency is associated with mental impairment in patients with constitutional Type I *NF1*

microdeletions.[41] However, none of the mosaic patients displayed mental impairments. This correlation indicates that the size of the deletion has tangible effects on phenotype, with mosaic patients typically containing smaller deletions than patients with inherited large deletions.[41]

Monozygotic twin studies have unraveled significant genotype-phenotype correlations. One case using cloned PCR products identified an *NF1* nonsense mutation at 4% VAF in buccal swabs and at 29% in blood in one twin who met full NF1 diagnostic criteria by the age of 3. In the other twin, who presented with two café-au-lait spots at age 3, no *NF1* variants were identified in blood, uroepithelial cells, or buccal swabs.[42] 4.1-6.9% of healthy, European children have two café-au-lait spots, so this finding was still consistent with absence of NF1 clinical features in the second twin. It was determined that the *NF1* mutation occurred after the twinning event, which in the case of dichorionic, diamniotic twins, usually occurs within the first 3 days post-fertilization, in the pre-morula stage. An alternative mechanism proposed to explain the lack of mutation in the second twin was reversion of the mutant allele to wild-type if the pre-twinning zygote harbored the mutation. This could occur due to mitotic recombination or gene conversion.[42]

Another case of monozygotic, monochorionic, diamniotic twins demonstrated a heterozygous *NF1* mutation in all tissues of one twin and mosaic levels of *NF1* PVs in the other who, by the age of 57, had not developed any overt features of NF1.[42] It was determined that the mutation still likely occurred before the twinning event, but in this case, the twinning even would have occurred 4-8 days post-fertilization. These two studies illustrate the extent to which timing of a mosaic mutation can influence phenotypic outcome and clinical heterogeneity.[42]

A study comparing digital PCR to MLPA for improved sensitivity in detecting *NF1* deletions detected 5 mosaic cases out of 46 DNA samples from NF1 patients.[28] Transmissibility of mosaic variants across syndromes is still not very well characterized. Interestingly, one study followed a Danish sperm donor who was later determined to be a gonadosomatic mosaic for NF1, with 20% variant allele frequency (VAF).[13] 23 of his offspring were identified and referred to the Centres for Rare Diseases for MLPA analysis. 9 were found to carry an *NF1* deletion of exons 15-29.[13] At the time of the report, only two genotype-phenotype correlations had been established for NF1 – microdeletions were known to be associated with relatively severe phenotypes and deletion of AAT in exon 17 associated with a milder phenotype.[13]

Two of the largest studies of mosaic-only NF1 patients were conducted in Spain and Toronto, Canada, and reported on 40[43] and 60 children[44] respectively with mosaic NF1. In the Spanish study, all children had café-au-lait spots with or without freckling while only 1 had cutaneous neurofibromas which overlapped with regions of pigmentary change.[43] All children lacked other clinical features of NF1. The mean age of diagnosis was 12 ± 2.57 years of age.[43] 15 patients had café-au-lait spots large enough to meet inherited NF1 diagnostic criteria, but all of these spots were limited to a specific body segment. This finding helps to distinguish mosaic from inherited cases in the event that café-au-lait spots meet the diagnostic criteria for inherited NF1.[43] In the Canadian study, 39 (65%) patients had localized pigmentary changes only, and 9 (15%) had neurofibromas only. The average age at diagnosis was 10.6 ± 4.6 years.[44] No cases of mosaic patients with juvenile xanthogranuloma have been reported, and only one mosaic patient with nevus anemicus has

been identified. No significant correlations could be drawn between the systemic manifestations seen (2 with epilepsy, 4 with mild scoliosis, 2 with speech disorders) and relationship to NF1. Only two patients developed tumors frequently associated with NF1, Hodgkin's lymphoma and stage IV abdominal ganglioneuroblastoma. Taking into consideration that these malignancies have never been reported in children, it is uncertain whether risk of cancer is truly associated with mosaic NF1.[43]

Type II-*NF1* deletions, which encompass 1.2Mb and are mediated by nonallelic homologous recombination between the *SUZ12* gene and its pseudogene, often occur postzygotically. At least 63% of all type II deletions are mosaic in NF1 and account for low-grade mosaicism (VAF < 15%).[35] This is likely correlated with the severe phenotype and increased risk of malignancy seen in full heterozygous patients with microdeletions. As of 2019, it was determined that FISH is the only method capable of detecting low grade mosaicism between 1-5% for *NF1* deletions.[35] Previously, MLPA used for the detection of *NF1* deletions was limited by a detection limit of 10-20%.[35] Only 3.4% of type-I microdeletions were shown by MLPA to be mosaic in a study analyzing 116 patients with Type I NF1 deletions.[35] In another study, ultra-deep sequencing of blood samples from 20 patients with type 1 *NF1* deletions did not detect allele frequencies >1% in any of the samples, confirming the low frequency of mosaic type I deletions. Up to 60% of atypical deletions likely exhibit mosaicism.[35]

Most recently, a retrospective chart review of 68 mosaic pediatric patients with at least one segmental or unilateral manifestation of NF1 was conducted.[45] They were diagnosed at an average age of 8.28 ± 4.47 years. This cohort followed established patterns of mosaic phenotype, with 72% presenting with only café-au-lait macules. Therefore, it is

recommended that identification of unilateral or bilateral pigmentary changes confined to a particular skin region or distribution should comprise clinical diagnostic criteria for mosaic NF1. At this point, only 15 mosaic patients have undergone genetic testing, 12 of which were found to have a genetic mutation.[45] As café-au-lait macules are commonly the only feature seen in mosaic cases of NF1, skin biopsies from hyperpigmented tissue should be analyzed for *NF1* mosaic mutation.[45]

NF2 – Neurofibromatosis type 2

Pathogenic variants in *NF2*, on chromosome 22q12.2 (OMIM®. Johns Hopkins University, Baltimore, MD. MIM Number: {101000, 607379}), cause type 2 neurofibromatosis, which affects approximately 1 in 33,000 births[32] with a nearly complete penetrance of 0.95.[33] The functional *NF2* gene product, merlin, a 595-amino acid protein, is a membrane-cytoskeleton scaffolding protein and critical regulator of contact-dependent inhibition of proliferation.[46] Interestingly, similarly to *NF1* mutation, one consequence of loss of *NF2* function is cell-proliferation via constitutive activation of the Ras/MEK/ERK pathway, but in NF2, this does not result in impaired learning and memory formation as in Type 1 neurofibromatosis.[47]

The hallmark feature of constitutional NF2 is bilateral vestibular schwannoma (VS), or schwannomas on both vestibulocochlear nerves, the presence of which establishes a definite NF2 diagnosis. Other benign tumors seen in NF2, in order of frequency, include unilateral vestibular schwannoma, subcutaneous schwannoma, intracranial meningioma, peripheral nerve schwannoma, ependymoma or astrocytoma, and schwannomas of other cranial spinal, and cutaneous nerves (**Figure 5**).[32] Ocular manifestations include early onset

cataracts, optic nerve sheath meningiomas, retinal or pigment hamartomas, and epithelial retinal membranes. Cutaneous manifestations include flat dermal schwannomas, called NF2 plaques, and spherical or ovoid subcutaneous nodular schwannomas.[47] More than 6 café-au-lait spots in an NF2 patient are rare. While the tumors listed previously are benign, schwannomas may rarely undergo malignant transformation (**Figure 5**).

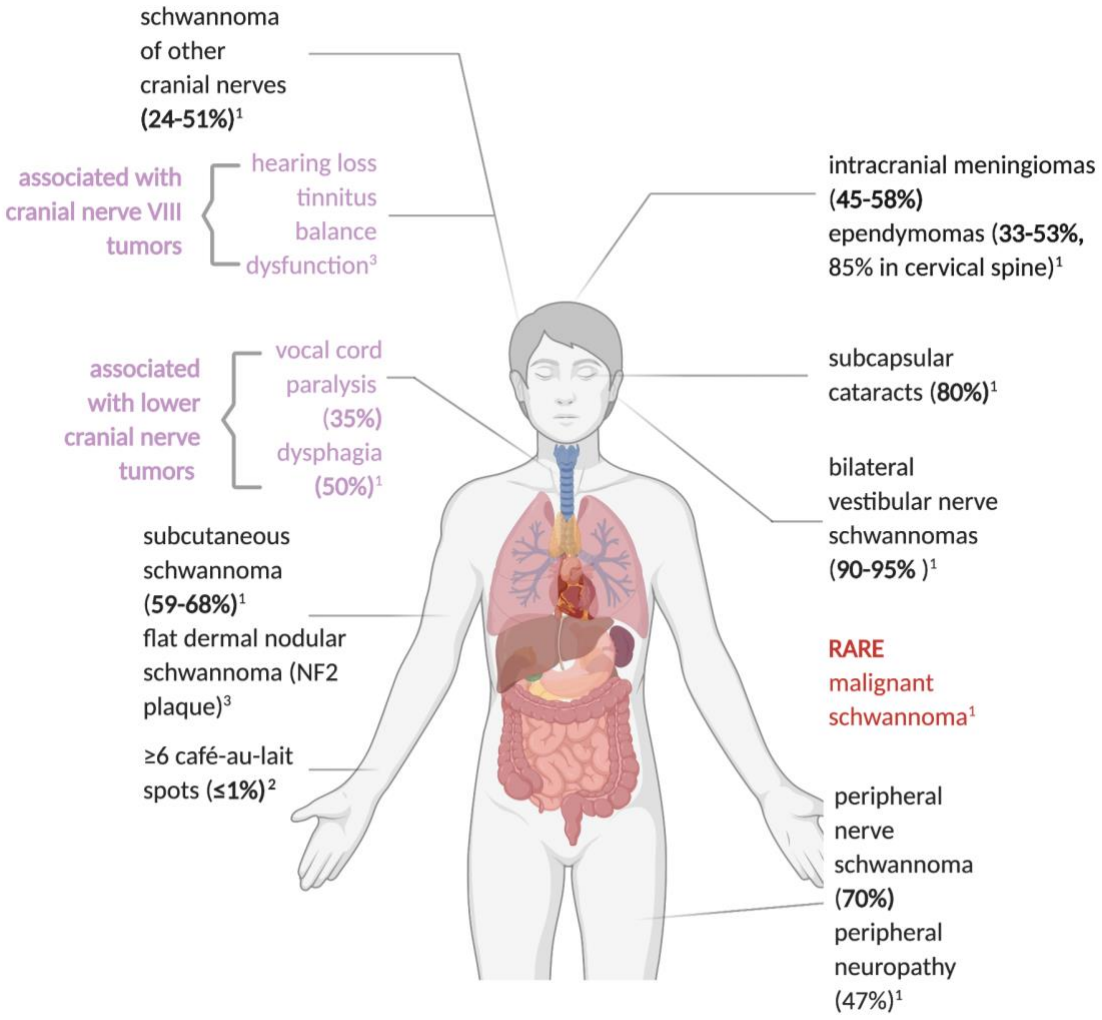

Figure 5 | Clinical features of Type 2 neurofibromatosis. Malignant tumors are noted in red. Purple text indicates associated symptoms. 1. Farschtschi S et al., Dtsch Arztebl Int., 2020; 2. Ruggieri M et al., Acta Otorhinolaryngol Ital., 2016; 3. Ruggieri M et al., Seminars in Pediatric Neurology, 2015

Following the Manchester diagnostic criteria, when classic bilateral VS is not observed, NF2 may be diagnosed if a patient has a first-degree relative with NF2, presents with unilateral vestibular schwannoma (VS) before 30 years of age, or possesses two of the following features: meningioma, glioma, schwannoma, juvenile posterior lens opacification or juvenile cortical cataract.[32] Tumor development in NF2 follows Knudson's two-hit model, with chromosome 22 often frequently incurring large deletions, and often involves loss-of-heterozygosity (LOH) of flanking and intragenic polymorphic markers.[48] Half of all cases are attributed to *de novo* mutations, and between 30-60% of these have been found to be mosaic. The mutation detection rate was 95% using MLPA and MPS in a study that found 76 NMIs out of 1361 mainly *de novo* patients meeting NF2 diagnostic criteria.[49] In patients with unilateral VS or multiple meningiomas, analysis of ependymoma tumors achieved 68% detection rate, the highest of all tissues sampled in one study.[49]

The age of onset for inherited cases, 22 years, is younger than the average for *de novo* cases, 34 years, owing to the typically milder features of sporadic cases.[49] The mean life expectancy for a patient with full heterozygous NF2 is 39 years.[50] The risk of transmission to offspring of patients with mosaic NF2 has been found to be between 8-12% depending on the parent's extent of mosaic variation.[33]

Over the last two decades, a number of large NF2 cohort analyses have identified significant proportions of mosaic patients. This review summarizes the most relevant findings of these studies below chronologically.

The first case of mosaic NF2 was recognized in 1994, when two NF2 patients were found to have the same C → T transition at site 169 in the *NF2* gene following analysis

using PCR-SSCP (polymerase chain reaction-single-strand conformation polymorphism) and heteroduplex analysis. However, the suspected mosaic patient presented with a significantly milder phenotype and was found to have a significantly weaker band intensity than the younger-presenting patient following analysis of blood genomic DNA. Buccal epithelial cells, hair roots, and urine epithelial cells from the suspected patient were found to carry the same variant at a frequency of <5%, indicating mosaicism. The methods used have a detection limit of 5-10%, so the true VAF is unknown. It was estimated that the mosaic patient had a VAF of 10% in blood. Site 169 was also found to be a potential mutation hot spot in NF2.[51]

One patient had a sister with bilateral VS and multiple small cranial tumors and a mother with late-onset NF2 but no *NF2* mutation identified. Linkage analysis showed that the patient had inherited the same maternal chromosome 22 as his sister and thus predicted a >99.9% risk of having NF2. However, dye primer sequencing of exon 8 to assess the degree of mosaicism in the mother and her two offspring showed that the patient matched the normal control and had not inherited the R262X mutation. The study suggests that the patient's mother had mosaic VAF present in her gonadal cells that were only transmitted to one of her offspring. The study also confirmed that linkage analysis can be a misleading method when determining inheritance of autosomal dominant genetic disorders.[52]

233 founder patients with NF2, defined as those without clinically affected parents, and bilateral VS were studied in 2002. SSCP and temperature gradient gel electrophoresis (TGGE) were used to analyze blood and tumor samples. Mosaicism was detected in the blood of 10 patients. 111 others had no mutation identified in blood, and of the 35 patients with tumor tissue available to analyze, 9 were determined to be mosaic based on identical mutations seen in multiple tumor types. 6 other patients demonstrating LOH of *NF2* in

tumors were deemed likely mosaics. A total of 48 mosaic cases were extrapolated from these results. Actual detection was confounded by limited tumor sample availability. The study estimated the frequency of mosaicism to be between 16.7% to 24.8% in founders with bilateral VS, established that mosaic cases of NF2 typically present at older ages but maintain the same eventual tumor development, and determined that better methods of detection are needed when tumor tissue is unavailable.[53]

In 2007, Evans et al. analyzed a database of 704 NF2 patients meeting Manchester criteria who were either referred to the lab for mutation analysis or who were part of their specialist service patient base.[54] 64 definite mosaic patients were identified, and using MLPA, the minimum proportion of mosaicism in classically-presenting NF2 cases was updated to be 33%, and 60% in individuals with unilateral VS. The mean age of presentation for mosaic patients was found to be 33.9 years, and risk of transmission estimated to be 1/12 for all NF2 patients with unilateral VS, and 1/8 in those with bilateral VS, based on the high proportion of mosaic cases in both categories.[54] In a follow-up study, 402 *de novo* NF2 patients with NMI in blood were analyzed in four age-stratified cohorts, and a more specific formula to assess risk of transmission was developed. The risk of transmission to offspring in a patient presenting with BVS at <20 years of age was found to be 29.3%, as compared to 5.5% in a patient presenting with asymmetric disease past the age of 40 since there is a 99% chance of mosaicism.[55]

Identifying methods to improve the sensitivity of detection of mosaic NF2 is still an area of investigation. In a study assessing the efficacy of amplicon sequencing in detecting low level mosaic variants, Sanger sequencing was not capable of validating frequencies

between 1.6% and 8.7% in known mosaic patients. Co-amplification at lower denaturation temperature (COLD)-PCR was additionally unable to detect VAFs below 1.6%.[56]

Of importance, mosaic NF2 bears different effects on patient mortality and life expectancy than inherited disease, parameters that may be predicted based on particular clinical features. An analysis of 1192 patients from the UK National NF2 registry, including 147 mosaic cases and 396 persistent NMIs concluded that mortality among mosaic patients was <1/3 that of patients with constitutional mutations. Additionally, those with splice-site mutations in exons 1-5 had a higher mortality rate than those with mutations in exons 6-15.[57] Whereas those with inherited disease experience a reduced average lifespan and higher mortality rate owing to tumor burden, malignancy, and perioperative complications, mosaic cases are associated with a reduced mortality rate.[47] Clinically, mosaic NF2 is now recognized by the presence of unilateral eighth-nerve schwannoma, associated ipsilateral meningioma, or multiple schwannomas localized to part of the peripheral nervous system,[47] and ultra-deep sequencing has been established as a method of detecting very low-frequency mosaic variants in both blood and tumor tissue – VAFs between 2.6 and 19.7% have been identified using MPS.[58]

In another analysis of 142 patients meeting the Manchester criteria registered to the Oxford NF2 Centre, 83 (58%) were determined to be mosaic following MPS performed on blood samples and on tumor tissue when available. The mosaic patients presented with a higher rate of unilateral VS (35.9%) than those with inherited disease (12.6%), while only over half of the mosaic patients presented with the classic bilateral VS, as compared to 96-100% of genetic cases with BVS.[59] These findings indicate that patients presenting with UVS

without other features of NF2 are possible candidates for mosaicism. Further research is necessary to establish a more definitive set of clinical features of mosaic disease.

A study including English children (N = 87) under the age of 18 diagnosed with NF2 found only 3 mild cases (12% of all mild cases) and 5 moderate cases (16% of all moderate cases) of mosaic pediatric NF2, with an average age of onset of 8.8 years for mild constitutional and mosaic cases. This stands in contrast to severe disease which has onset in infancy. Of note, 3.5% of all pediatric cases exhibited attention deficit hyperactivity disorder (ADHD) and autism-spectrum traits. An investigation of drug therapeutic effects in this cohort found that bevacizumab, given for treatment of rapidly growing unilateral and bilateral VS allowed patients to avoid surgical resection in some cases. Negative effects include hypertension and proteinuria. Everolimus shows limited efficacy and safety for treatment of overgrowth in NF2.[60]

Last year, two large cohort studies were conducted.[27, 61] Teranishi et al. aimed to establish better rates of mosaic diagnosis in 53 patients with *de novo* NF2 using targeted deep sequencing of DNA of a wide variety of tissue types including blood, buccal mucosa, hair follicle, and tumor. All patients fulfilled the Manchester criteria. Before this study, the detection rate of germline PVs in patients with a family history was 90%. The rate was 25-60% in *de novo* patients.[61] The genetic diagnostic rate of mosaicism was only 20.2% to 23.5% at the time of this study. This series saw a slightly improved rate. 24 patients (37.7%) were mosaic following direct Sanger sequencing and MLPA of blood and subsequent targeted MPS performed on other tissue samples. The growth rate of schwannomas differed between the mosaic and non-mosaic groups based on age of presentation, whereas growth rate of meningiomas did not. Previously, it was also determined that 60% of *de novo* cases may be

presumed mosaics. Multi-tissue analysis was proven to be effective at detecting low VAF. Timing of the mutation, however, remains ambiguous, as tissue samples are not homogeneous in terms of cell population.[61]

The most recent series used MPS to analyze 1055 patients with sporadic NF2. 232 proven and probable mosaic patients were identified with VAFs between 0.2-30%. Proven mosaicism was defined by a PV detected in lymphocyte DNA at VAF below 30% or by identical PVs in two anatomically distinct tumors. Probable mosaicism was defined by presence of two hits in a single tumor when only one tumor sample was available and no PVs identified in blood. Nonsense mutations were identified in 41.81% of mosaic cases, and the median age at diagnosis for mosaic patients was 35, as compared to 21 in non-mosaic patients.[27] 428 (40.57%) *de novo* patients were NMI. This is likely due to the low availability of tumor samples. Predictive tests conducted on children of mosaic parents with PVs in blood found 10.7% positive cases. None of the children of presumed mosaic patients who had no PV in blood tested positive for *NF2* variants.[27]

Diagnostic Overlap with Schwannomatosis

Mosaic NF2 has often been confused with schwannomatosis, a typically sporadic schwannoma-predisposition syndrome associated with mutations in *SMARCB1* or *LZTR1*. Diagnosis of schwannomatosis includes presentation > 30 years old, 2 non-intradermal schwannomas, at least one of which has been histologically confirmed, no evidence of VS after magnetic resonance imaging (MRI), and no constitutional *NF2* mutation.[32] In a study assessing diagnostic criteria for NF2, it was determined that 2/3 of schwannomatosis cases with unilateral VS and more than two nondermal schwannoma actually have NF2, a majority

of which may be mosaic.[49] However, in the case of mosaic NF2 with unilateral VS, if another NF2 feature is observed, the second tumor should contain an identical mutation to the one seen in the VS. [46] Overall, about 50% of apparent schwannomatosis cases without an *LZTR1* or *SMARCB1* variant are mosaic cases of NF2.[49]

TSC1/TSC2 **– Tuberous Sclerosis Complex**

Tuberous sclerosis complex (TSC) is characterized by seizures, intellectual disability, and autism, as well as by variable tumor development involving the skin, heart, brain, kidneys, lungs, and other organs.[62] Two-thirds of TSC cases are sporadic.[63] It is fully penetrant, affects 1/6,000 – 1/10,000 individuals, and is caused by inactivation of either *TSC1,* on chromosome 9q34.13, or *TSC2,* on chromosome 16pq13.3 (OMIM®. Johns Hopkins University, Baltimore, MD. MIM Number: {191100, 191092}). *TSC1,* composed of 23 exons, encodes the protein hamartin, and *TSC2*, composed of 43 exons, encodes tuberin, which inhibits mTOR when in complex with the *TSC1* gene product. Loss of function of either *TSC1* or *TSC2* results in hyperactivation of the mTOR pathway. Variants in *TSC1* constitute 26.4% of pathogenic variants (PVs) while variants in *TSC2* are more common in both germline and somatic TSC, comprising 73.6% of all pathogenic variants.[9]

The hallmark feature of TSC is the kidney angiomyolipoma, a benign tumor composed of abnormal vasculature and immature smooth-muscle and fat cells, seen in 55-75% of patients.[9] Major features include at least three hypomelanotic macules at least 5 mm in diameter, at least three angiofibromas or fibrous cephalic plaques, at least two ungual fibromas, shagreen patch, multiple retinal hamartomas, cortical dysplasias including tubers and cerebral white matter radial migration lines, subependymal nodules (SEN),

subependymal giant cell astrocytoma (SEGA), cardiac rhabdomyoma, lymphangioleiomyomatosis, or at least two angiomyolipomas. Minor features include "confetti" skin lesions, at least three dental enamel pits, at least two intraoral fibromas, retinal achromic patch, multiple renal cysts, or nonrenal hamartomas (**Figure 6**).

Definite diagnosis of TSC is made when two major features, one major feature with two or more minor features, or a pathogenic variant in *TSC1* or *TSC2* is seen.[9] The most common causes of mortality in patients with TSC are bleeding from an angiomyolipoma or enlargement of subependymal nodules into SEGA,[62] benign tumors that can obstruct cerebrospinal fluid drainage leading to obstructive hydrocephalus.[9] Tumor formation in TSC relies on loss of heterozygosity. *TSC1*-associated tuberous sclerosis typically presents with a less severe phenotype, owing to the decreased frequency of second-hit events. These cases usually have lower tumor counts in multiple organs and lower cortical tuber counts. On the contrary, cases of *TSC2*-associated tuberous sclerosis usually have earlier onset of seizures that are more refractory and harder to treat and represent a higher proportion of patients diagnosed with infantile spasms and higher rates of autism spectrum disorder and intellectual disability.[9]

mTOR inhibitors such as everolimus have proven to be effective in reducing SEGA size and growth and in reducing tumor burden of renal angiomyolipomas and skin lesions in adults. However, these benefits stop with cessation of treatment. Additionally, children with TSC, especially those under the age of 6, benefit from decreased seizure frequency after treatment with everolimus. Undesirable side effects, including stomatitis and increased risk of infection, point to the continued necessity of identifying therapeutic options for TSC.[9]

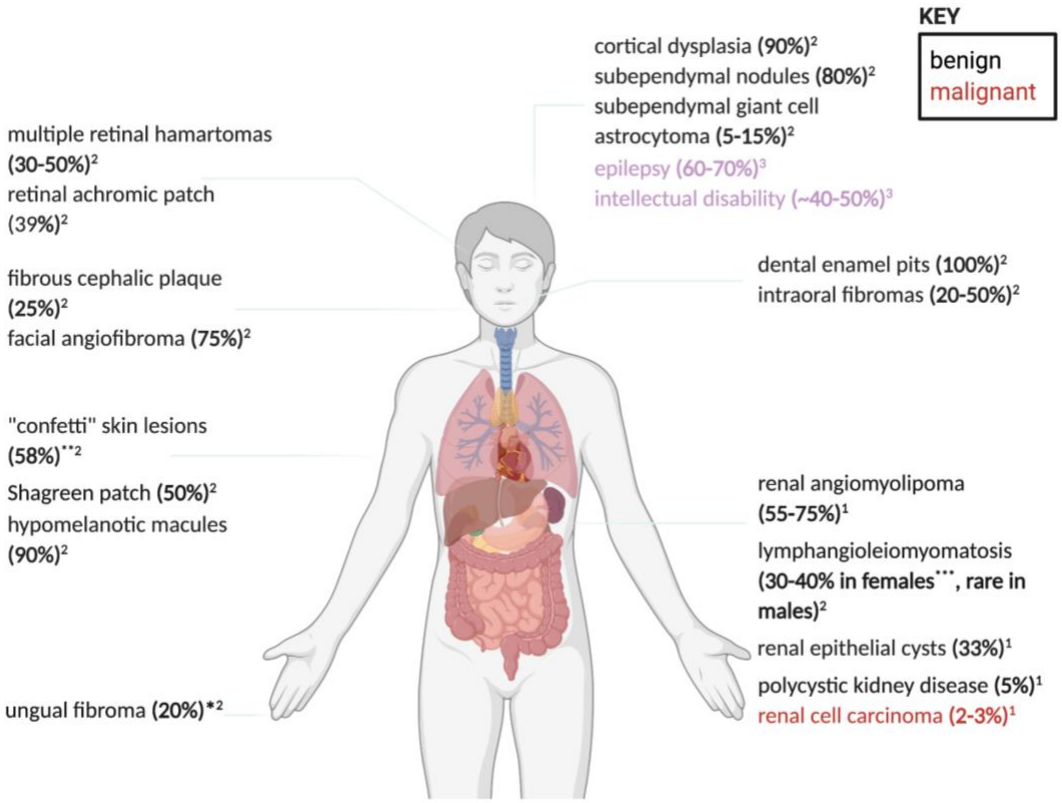

Figure 6 | Clinical features of Tuberous Sclerosis Complex. The brain is the most severely affected organ in TSC. Brain lesions are often accompanied by epilepsy, developmental delay, or neuropsychiatric disorders such as autism spectrum disorder. While SENs are typically asymptomatic, they can grow into SEGAs, which can be a cause of fatality due to onset of obstructive hydrocephalus.[9] Renal cell carcinoma is the only malignant manifestation of TSC. *can be as high as 80% in older population **3% in children ***can be up to 80% in females by age 40 1. Salussolia CL et al., Annu Rev Genom Hum Genet., 2019 2. Northrup H et al., Pediatr Neurol., 2013

Gonadal mosaicism has been observed in both *TSC1* and *TSC2*-associated Tuberous Sclerosis Complex. In a study of 62 unrelated families with mutations in either *TSC1* or *TSC2,* 6 families had affected members with mosaicism. Five had parents with mild mosaicism that was transmitted as full-TSC to offspring, while 1 family had 3 children with TSC, but clinically unaffected parents who were found to be mosaic for TSC following

single-strand conformation polymorphism (SSCP) analysis and direct sequencing. Two families had mutations in *TSC1* and four in *TSC2*. Clinical features in the children of all mosaic families were of variable severity. From this study, the frequency of gonadal or somatic mosaicism was estimated to be around 10%.[62] Multi-tissue sampling has been done to identify mosaic TSC for many years. One case study using heteroduplex analysis found an inactivating *TSC1* variant in exon 15 present in one third of leukocytes that was completely absent from the buccal mucosa. Mutant allele frequency was determined to be 0.35 in blood, with fractions ranging from 0.12 to 0.42 in urine, hair roots, and buccal mucosa. Despite mosaic status, the patient had severe clinical manifestations.[63] Low VAFs in severe TSC could reflect an alternative mechanism of gene inactivation such as epigenetic silencing. The study's results also suggested that hamartomatous lesions can occur even if only a small proportion of cells in an organ are mutated. Involvement of other driver mutations must be investigated. Another family study found the rate of mosaicism to be 6% based on clinical findings in children diagnosed with TSC in 7 families born to unaffected parents. The recommended risk of transmission was roughly estimated to be 2-3% for parents with apparently sporadic TSC.[64]

The mutational spectrum of TSC has been well studied.[65,66] In a study of 224 index patients from pediatric neurology practices in the US and Poland, it was determined that nonsense, splice-site, and missense mutations make up around half of all TSC-causing variants and that small deletions and insertion comprise around 38%. Large genomic deletions and rearrangements are rare and have been reported only in the *TSC2* gene in TSC. In general, *TSC1* is less frequently mutated than *TSC2* and is associated with a milder clinical phenotype.[65] More recent findings have found that patients with *TSC2* mutations are

diagnosed an average of 9 years earlier than those with *TSC1* mutations and 11 years sooner than those with NMI status.[67] If a patient has forehead fibrous plaques, liver angiomyolipomas, retinal hamartoma, or significant renal involvement, it is highly likely that they have a *TSC2* mutation. This is based on findings that 104/224 (84%) of patients presenting with these features had a confirmed *TSC2* mutation following leukocyte analysis with denaturing high-performance liquid chromatography (DHPLC). Significant renal involvement is rarely seen in patients with *TSC1* variants. Interestingly, the 38 (17%) patients who were persistent NMIs had significantly milder features than the *TSC2* mutant group which differed slightly from those seen in the *TSC1* group.[65] It is likely that mosaicism accounted for a large proportion of the NMI group.

In a follow-up study, the 38 NMI patients' blood DNA were re-analyzed using ultra deep pyrosequencing.[68] Only 2 (6.1%) cases of mosaicism were found. It was previously shown that mosaicism is more frequently seen with genomic *TSC2* deletion mutations than with smaller *TSC2* indels and point mutations. Additionally, mosaicism has been seen in 27% of patients with combined TSC2-polycystic kidney disease syndrome, which is traced back to genomic deletions of parts of both *TSC2* and the *PKD1* gene. The study concluded that mosaicism is rare in TSC with persistent NMI status. However, alternative methods with the capability to detect VAFs below 2% are necessary to validate this conclusion.[68]

A recent study investigated rare cases of TSC patients presenting with tubers but no SENs (3/220) as well as no mutations identified in either *TSC1* or *TSC2*.[69] Cases of TSC patients without co-occurring SENs and cortical tubers are very rare. In those with only one or a few tubers but no SENs, a postzygotic mosaic mutation in the neuroectoderm may explain its limited distribution and clinical manifestations. In light of this study, it is clear that

localized somatic mosaicism may also exist in TSC. However, further genetic analyses of patients fulfilling this profile are necessary.[69]

TSC mosaicism was one of the first genetic syndromes to be analyzed for low level VAF using MPS. Of 45 TSC patients with NMI status following conventional sequencing, 26 (58%) were shown to be mosaic following MPS. Additionally, intronic mutations were identified in 18 (40%) of the NMI cohort. DNA from blood, normal skin, saliva, angiofibroma, and lesioned skin biopsies was analyzed. VAF in the mosaic group ranged from 0.21% to 34%. Seventeen patients' VAF fell below 5%, five patients had VAF below 1%, and two patients had variants detectable only in skin tumor biopsies. While these findings have shown that the frequency of mosaicism in TSC is higher than previously thought, only 21 of the samples were validated by secondary sequencing methods, e.g. Sanger sequencing, single nucleotide sequencing, PCR, and amplicon MPS.[4] The existence of a third TSC gene was once speculated, but MPS and tumor tissue analysis have made it clear that this is unlikely, as mosaicism has now been observed in a significant proportion of TSC cases.[4,66]

The most recent analysis of 39 TSC patients with NMI status evaluated blood DNA as well as lesioned skin biopsies, normal skin, saliva, cultured cells, urine, and semen using targeted MPS. *TSC1/TSC2* mutations were verified using amplicon MPS. As expected, the patients with the lowest variant allele frequencies had fewer TSC features than those with higher VAFs. Importantly, biopsies from skin lesions contained a 2-fold higher mosaic VAF than was found in blood, normal tissue and saliva. Shagreen patches and ungual fibromas' high VAFs indicate high tumor purity in these lesions. Numbers of mosaic patients based on level of variant allele frequency were estimated based on the study's findings. At VAFs below

3%, there were significantly fewer mosaic cases. At increasingly lower VAF levels, the model predicts increasingly greater numbers of mosaic patients. Considering this, many cases of mosaic TSC at very low VAFs may still be undetected. While it was shown that semen analysis can be predictive of risk of transmission of a mosaic variant to offspring, it is still unclear how VAF in other tissues correlates with transmission risk.[70]

Most renal angiomyolipomas and SEN/SEGAs demonstrate copy-neutral (CN) LOH at approximately 95% and 85% respectively .[66] However, one study identified second-hits in only 35% of cortical tubers. Epigenetic changes may be a possible alternative mechanism to explain lesion formation, but no instances have yet been seen. It may be the case that a heterozygous mutation is sufficient for the development of some TSC-associated lesions.[66]

Conclusive correlations between mosaicism and specific clinical features have not been established for TSC, but it is known that patients with adult-onset features, asymmetric facial angiofibromas, and absence of SENs or cortical tubers may be indicative of mosaicism.[71] Localized germline mosaicism in TSC has been identified in a clinically-unaffected Japanese father who had two children with *TSC1* variant-associated cardiac rhabdomyomas, a feature predictive of and almost exclusive to TSC.[72] In a cohort of 94 infants diagnosed with TSC, cardiac rhabdomyomas were identified in 97%.[73]

The current rate of mosaicism among TSC patients is estimated to be 10-15%. It is also clear that childhood-onset mosaic TSC occurs at a significant rate. The EPISTOP study (a long-term, prospective study assessing clinical and molecular biomarkers of epileptogenesis in TSC) which enrolled 101 children diagnosed with TSC before the age of 4 to study clinical and molecular biomarkers of epileptogenesis, includes 9 cases of mosaic

TSC with VAFs ranging from 0.7% to 32%, all of which are mosaic in the *TSC2* gene. Despite established evidence that *TSC2* variants are associated with more severe and greater numbers of TSC features, these mosaic cases presented with milder phenotypes and improved seizure-free survival.[73]

Recently, a diagnostic algorithm for better detection of adult mosaic TSC was proposed to expedite diagnosis and counselling. It was suggested that those with onset of ungual fibroma before the age of 15, angiofibromas before 5, and 3 or more mucocutaneous findings (MCF) with SENs, represent likely germline TSC. These patients should first undergo blood DNA analysis. On the other hand, mosaicism may be suspected in patients who present with less than three MCFs, absence of tubers and SENs, and asymmetrical AFs with fewer than 100 lesions. In these cases, MPS should be performed on available TSC tumors and be followed by amplicon MPS on other tissues.[74] In 2021, one new case of TSC has been noted – a pediatric patient with daily seizures starting at 2 years old, SENs on the right ventricle wall, and multiple hypomelanotic macules. A mosaic *TSC2* variant was identified in a cortical tuber sample at 3% allele frequency using whole exome sequencing (WES) and targeted MPS. No variants were identified in blood or normal tissue, highlighting the importance of lesioned tissue analysis in TSC patients with NMI status.[75]

***PTEN*-hamartoma tumor syndrome**

PTEN-hamartoma syndrome (PHTS) is a family of syndromes caused by mutations in the phosphatase and tensin homolog gene (*PTEN). PTEN*, on chromosome 10q23.31, encodes a dual-specificity phosphatase that inhibits cell spreading via dephosphorylation of focal adhesion kinase (FAK) and regulates downstream effects of the PI3K pathway by

dephosphorylating phosphatidylinositol 3,4,5-triphosphate (PIP3) **(Figure 2)**. Under the broad title of PHTS are Cowden syndrome (CS), Bannayan-Riley-Ruvalcaba Syndrome (BRRS), Proteus syndrome (PS), and Proteus-like syndrome.

Cowden Syndrome

CS (OMIM #158350) is a hamartoma syndrome characterized by trichilemmoma and mucocutaneous papules (**Figure 7**) that affects approximately 1 in 200,000 to 1 in 250,000 individuals.[76] Diagnosis of CS is based on presence of two major criteria (see ref. 77): Lhermitte-Duclos disease (LDD), thyroid carcinoma, macrocephaly, or breast cancer. One must be either LDD or macrocephaly.[77] 30-35% of CS cases that meet clinical diagnostic criteria have a detectable *PTEN* mutation.[78]

Bannayan-Riley-Ruvalcaba Syndrome

BRRS (OMIM #158350) is a congenital syndrome characterized by macrocephaly, intestinal hamartomatous polyposis, vascular malformations, lipomas, and genital freckling.[17] 60% of BRRS patients have a *PTEN* mutation.[78] No other BRRS-causing genes are known.[78] 11% of BRRS cases are caused by *PTEN* deletions that sometimes also encompass the *BMPR1A* gene on chromosome 10q23.3.[79]

Proteus and Proteus-like Syndrome

PS (OMIM #176920, 164730) is a tissue overgrowth syndrome characterized by hemihypertrophy, macrocephaly, cerebriform connective tissue nevi, epidermal nevi, and lipomatosis that arise postnatally,[17-80] and interestingly, mosaic distribution of lesions,

progressive course, and sporadic onset are diagnostic criteria.[17] MPS performed on epidermal nevi, vascular malformations, and overgrown muscle samples have shown the best rates of detection of mosaicism in PS.[11] Proteus-like syndrome was suggested to be part of PHTS in 2000.[17] Overall, PHTS increases lifetime risk of developing breast, thyroid, endometrial, kidney, colon cancers, and melanoma.[15]

Between 11-48% of all PHTS cases can be attributed to *de novo PTEN* variants.[30,79] It has been demonstrated that hamartomas may develop in individuals heterozygous for *PTEN* variants without loss of the second functional allele.[81]

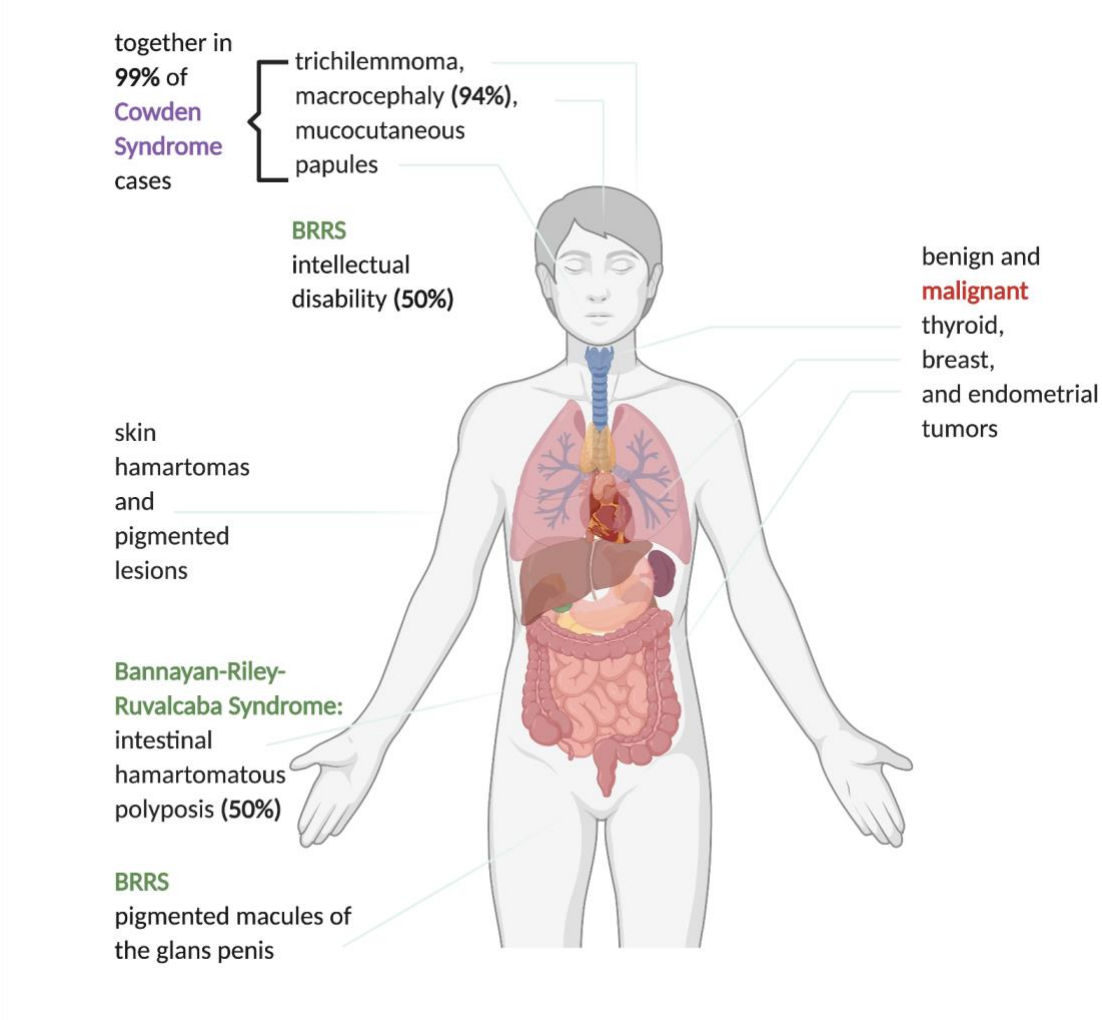

Figure 7 | *PTEN*-hamartoma tumor syndrome clinical features. PHTS diseases share some common clinical features. CS and BRRS each possess additional unique features that aid in distinguishing between the various *PTEN*-associated syndromes. Frequencies for some clinical features are not listed or found in the literature. Malignant tumors are indicated in red text. Zhou et al., Human Molecular Genetics, 2000.

Mosaicism in PHTS is not well documented. In recent years, no large cohort studies have been conducted specifically analyzing mosaic cases of PHTS. In cases of inherited heterozygous *PTEN* syndromes, mosaic second-hit variants have been seen at allele frequencies below 10%.[82] Frameshift and nonsense variants comprise the majority of the few

mosaic cases that have been identified.[17,83,30] Only two cases of mosaic deletions involving *PTEN* have been described.[84,79]

Four mosaic Cowden syndrome cases have been documented.[30,76,83,85] Up to 85% of CS cases are thought to be caused by germline *PTEN* mutations.[76] The first case of a mosaic *PTEN* variant causing CS was only recently identified when a 20-year-old woman presented with a history of gastrointestinal problems and no other features of CS. Her father, who had Hashimoto's thyroiditis but no cutaneous signs of PHTS, was subsequently found to carry a mosaic *PTEN* variant in blood at a frequency of less than 10% using peak height ratios. Both he and his daughter exhibited only mild features of CS. The father's precise variant allele frequency was not determined in the study.[83] Because of the rare possibility of mosaicism in PHTS, single sperm analysis for haplotyping was recommended. In the second documented case of mosaic CS, a father with one healthy child requested preimplantation genetic diagnosis (PGD) after CS diagnosis at the age of 32. Sanger sequencing found a *PTEN* variant in exon 7 at <20% VAF in the father who subsequently had a second child who inherited a mutant allele. PGD is susceptible to allele-drop out, in which only one allele copy of the DNA analyzed may be amplified. This especially poses a challenge in accurate counseling for mosaic cases.[85]

One of the most thorough mosaic CS case studies to date used MPS to identify a *PTEN* variant at 1.7% VAF in peripheral blood leukocytes. The patient had Lhermitte-Duclos disease, which is indicative of CS, along with macrocephaly, parietal stroke, mucosal papillomas, multiple keratoses, and hamartomatous polyps and ganglioneuroma. Samples from endocervical mucosa with squamous metaplasia and from normal colonic mucosa contained VAFs of ~25%, and a biopsy from a dysplastic gangliocytoma contained

heterozygous VAF levels. Her son had no features of *PTHS*. As evidenced by this case, mosaic CS may not exclusively give rise to a mild phenotype as is typically the case in mosaic TSSs.[30]

Finally, a fourth case of presumed mosaic CS due to a partial deletion variant was identified using array-based comparative genomic hybridization (array-CGH) in a woman meeting CS diagnostic criteria. The deletion included exons 6-9 and was identified in peripheral blood at 47 ± 10% frequency. Again, VAF was not determined in other tissues, so it is unknown whether the patient truly had VAFs consistent with mosaicism. In keeping with findings from the previous case study, this patient's phenotype was of comparable severity to inherited cases, as she met two major and five minor diagnostic criteria. Based on the high VAF, it is likely that the mutation was acquired in the early stages of postzygotic cell division.[76]

Two cases of mosaic BRRS have been identified due to mosaic 10q23 deletions. A French study analyzed four children with juvenile infantile polyposis. Macrocephaly and slight facial dysmorphia in three of the patients and lipomas and hemangiomas in the fourth led to consideration of a BRRS diagnosis. While all children were initially negative for *PTEN* point mutations, a mosaic deletion involving *PTEN* and *BMPR1A* was identified in peripheral blood at a VAF of 17% in one child and validated using FISH.[86] In the second case, the patient was suspected of having BRRS at 9 years old, but Sanger sequencing, MLPA, and array-CGH analyzing *PTEN* were all negative. When he presented for resection of his thyroid gland for multinodular goiter, BRRS diagnosis was confirmed, and re-analysis of a colonic polyp, buccal mucosa, and blood uncovered mosaic VAFs at $\log_2$-ratio -0.20 in buccal mucosa and <10% VAF in blood. MLPA showed a reduced signal for *PTEN,*

KLLN, and *BMPR1A* in a thyroid sample and in the colonic polyp sample, indicating presence of a 10q23 deletion.[79]

In an early study assessing whether Proteus-like syndrome could be classified under the umbrella of PHTS, an individual presented with a germline *PTEN* R335X mutation in exon 8. His parents' wild-type *PTEN* status confirmed that the subject carried a *de novo* mutation. PCR-based denaturing gradient gel electrophoresis (DGGE) performed on biopsies from a nevus, lipomatous mass, and arteriovenous malformation identified a second-hit mosaic mutation in a different exon. The patient was deemed to be simultaneously germline heterozygous for the R335X mutation and somatic mosaic for the R130X mutation in *PTEN*. [17] A similar second-hit "exacerbation" of heterozygous germline disease was seen in a patient presenting with an atypical case of Cowden syndrome who in addition to fulfilling typical CS criteria exhibited additional features of PS including segmental overgrowth, arteriovenous and lymphatic vascular malformations, lipomatosis, and linear epidermal nevus. The patient carried a CS-causing germline variant in exon 5 that was identified in blood and biopsied lesions using PCR-based DGGE. Noting a decreased signal for the wild type allele in a cutaneous fibroma, an epidermal nevus, and a lipoma, microsatellite typing was performed on these tissues which confirmed LOH.[80] In light of these cases, it is clear that both heterozygous and second-hit mosaicism are possible in disorders like PHTS whose overgrowths and lesions do not rely on LOH.

In further support of the notion that mosaic PHTS does not necessarily correlate with a mild phenotype, one case of a mosaic deletion involving *PTEN* presented with more severe intellectual disability than a germline *PTEN* patient analyzed in the same study. The mosaic patient's variant was enriched in the thyroid gland and in a benign multinodular

goiter and was ultimately diagnosed with mosaic BRRS. Previous evidence has also suggested that correlations cannot be drawn between the size of the deletion and the severity of clinical symptoms. However, the deletions identified in these two patients encompassed over 40 genes. Thus, genotype-related, graded, clinical severity cannot be ruled out for the *PTEN* gene itself.[79]

Mosaic *PTEN* variants resulting in features that are not consistent with or that do not meet diagnostic criteria for PHTS have also been documented.[81] A young boy presenting with autism spectrum disorder (ASD), determined to be Asperger's, and a small congenital retro auricular hamartoma without macrocephaly was initially suspected of having NF2 or schwannoma predisposition syndrome. These diagnoses were ruled out following MPS screening on blood, underscoring the importance of highly sensitive techniques in detecting low frequency variants. Ultimately, the patient was diagnosed with glioneuronal hamartoma due to heterozygous mosaic variants that likely occurred at the end of the third week post-conception.[81]

VHL – Von Hippel-Lindau Syndrome

Mutations in the *VHL* gene on chromosome 3p25 can cause a benign and malignant tumor predisposition syndrome called Von Hippel-Lindau (VHL, OMIM #608537). VHL is seen in 1/45,000 individuals, has 90% penetrance by age 60, and may be first diagnosed at any age.[87] Around 80% of VHL cases are familial, with the rest being sporadic.[88] The two protein products of the *VHL* gene act in the oxygen-sensing pathway, microtubule stability and orientation, cilia formation, aging, cytokine signaling, collagen IV regulation, and extracellular fibronectin matrix assembly.[87]

VHL causes hemangioblastomas of the brain, spinal cord, and retina, renal cysts and clear cell carcinoma, pheochromocytoma, pancreatic cysts, neuroendocrine tumors, endolymphatic sac tumors, and epididymal and broad ligament cysts (**Figure 8**). VHL is divided into Type 1, which carries a low risk of pheochromocytoma and Type 2, accompanied by a high risk of pheochromocytoma. Type 2 is subdivided into Type 2A, which carries a low risk of RCC, Type 2B, with high risk of RCC, and Type 2C, with risk of pheochromocytoma only.[89] Pathogenic missense mutations are often responsible for Type 2 VHL.[90] Patients are diagnosed with VHL if they present with two of these tumors, including at least one hemangioblastoma, or with one tumor and a family history of VHL.[91] Complications from cerebellar tumors include headache, vomiting, disturbed gait, or ataxia, pain from spinal hemangioblastoma, or sensory and motor loss due to cord compression. Vision loss is sometimes a result of retinal hemangioblastoma, a typical initial manifestation of VHL. The leading cause of mortality from VHL is malignancy of a renal cell carcinoma.[90]

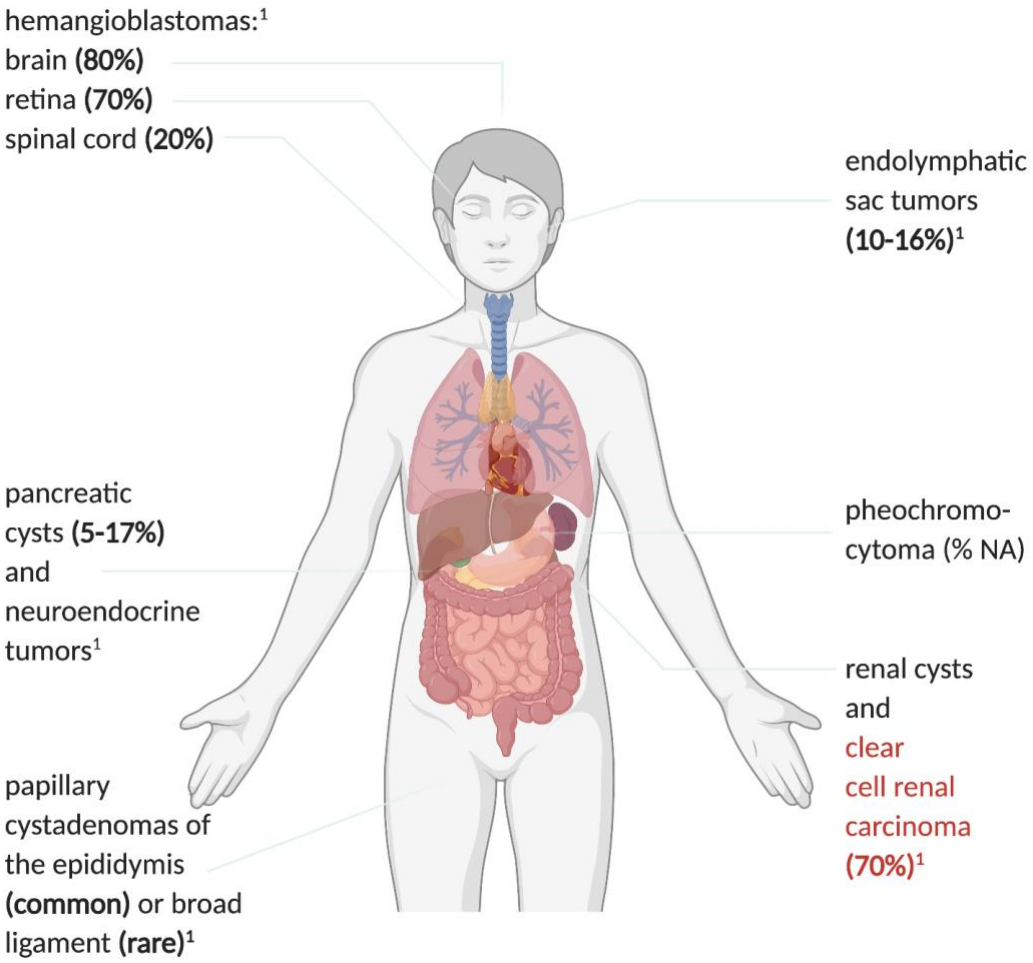

Figure 8 | Von Hippel-Lindau Syndrome. Malignant tumors are indicated in red text. 1. van Leeuwaarde et al., GeneReviews, 2018.

About 992 unique *VHL* mutations with known pathogenicity have been recorded (VarSome database-updated March 30, 2021, Gene: *VHL*).[16] 5% of VHL cases have been classified as no mutation identified due to under-detection by conventional sequencing

methods, and mosaicism may account for a significant portion of these cases.[91] VHL often results in highly vascularized tumors owing to the functional gene's role in degrading hypoxia inducible factor-1α (HIF1α). When *VHL* is mutated, HIF1α levels are high, leading to high vascularization in the respective tumors.[88]

Recently, a protocol to identify mosaic VHL was proposed based off of the results of the largest study ever conducted analyzing VHL patients with no mutation identified. MPS was performed on DNA from the peripheral blood mononuclear cells of forty-seven patients who screened negative for *VHL* variants but who had phenotypes evocative of VHL. Forty-six cases were sporadic and one familial. Digital droplet PCR, which has a threshold rate of 1% VAF, was used as a validation method. Four mosaic cases were identified (8.5%), all of whom presented with a greater median number of lesions than the remaining cohort. This could either mean that mosaic cases present with a more severe phenotype or that the remaining cases contained even lower frequencies of variant alleles.[91] Of note 23 patients from this series had only one VHL feature. The four mosaic patients met diagnostic criteria, giving a rate of mosaicism of 16.6% among those with typical VHL phenotypes.[91]

It has been suggested that in contrast to mosaic cases of other autosomal dominant diseases, a mosaic case of VHL does not necessarily imply a milder phenotype.[92] One of two mosaic patients identified out of a group of eight VHL cases with a VAF of 5.7% presented with early onset severe features – a central nervous system hemangioblastoma, right pheochromocytoma, pancreatic endocrine tumor, small adrenal nodule, and unilateral renal cell carcinoma (RCC), with the first tumor diagnosed at 16 years old.[92] In another case study, a patient presenting with bilateral RCC and diffuse renal cysts at the age of 65 could not

undergo radical nephrectomy and died a year after diagnosis. She was found to carry a mosaic *VHL* mutation at 18.8 ± 3.84% VAF in blood. In cases like these, the presence of pathogenic variants in other genes must be considered. Another gene, *SWI/SNF* (SWItch/sucrose non-fermentable), is often mutated in RCC.[93] It is worth investigating whether it is frequently included in deletions involving *VHL*.

By contrast, several mosaic VHL cases with milder phenotypes have been reported.[88,94] The first reported case of VHL mosaicism, an exon 3 variant, was detected in buccal mucosa samples using SSCP in a clinically asymptomatic mother who bore a son with full blown disease. She was diagnosed at age 48 and found to have pheochromocytoma and renal cysts with no LOH.[94] Santarpia *et al.* performed DHPLC analysis on peripheral blood lymphocytes, buccal mucosa, hair roots, and skin fibroblasts in a father suspected of mosaicism after his daughter was diagnosed with full heterozygous VHL. At 51 years of age, he was found to carry an exon 3 variant at 15% VAF only in buccal, hair, and skin fibroblast samples, and only presented with angioma of the glans penis and a renal cyst. He had undergone surgery for epididymal cystadenomas and a mandibular cyst at 43 years old. VHL was undiagnosed at the time.[88]

One of the oldest and most highly cited studies on VHL mosaicism evaluated 42 first generation VHL patients from 181 VHL cases documented by the National Institutes of Health (NIH).[95] Two patients were found to have a mosaic parent, giving a rate of 4.8% for likelihood of mosaic parents in sporadic cases with no documented family history.[95] In the first of these families, the mother was found to have mild disease features, including a small hemangioblastoma, several pancreatic cysts, and a single renal cyst. Interestingly, fluorescence *in situ* hybridization (FISH) detected a *VHL* deletion in 47% of B-cell

lymphocytes. In the second family, the father had late-onset advanced disease features, including clear-cell RCC, renal and pancreatic cysts, and hemangioblastomas of the cerebellum and lower brain stem. A deletion was determined via direct sequencing and conformational sensitive gel electrophoresis (CSGE) analysis of buccal cell and skin fibroblast DNA.[95]

More recently, a retrospective cohort study in Denmark evaluated 122 diagnosed VHL patients, 8 of whom were mutation-negative, and 68 assumed VHL patients who had no family history of VHL but who had at least two VHL-related clinical manifestations, fulfilling Danish diagnostic criteria. Medical histories were available for 135 out of 167 (83%) of the diagnosed patients' first-degree relatives and 205 of 331 of the assumed patients' first-degree relatives. Because not all of the assumed VHL patients had undergone genetic testing, their clinical features were compared to those of the diagnosed patients to determine likelihood of having diagnosable VHL. 22 patients were estimated to have *de novo* variants, a majority of whose parents must still be tested for germline mosaicism.[96] The lack of conclusive genetic evidence of mosaicism within these larger cohorts, along with recent evidence about the success of MPS in this field suggests that these cohorts of clinically-suspected VHL may benefit from re-analysis with more sensitive screening methods.

RB1 **– Retinoblastoma**

Retinoblastoma (Rb) is a malignant pediatric tumor of the retina seen in children less than 7 years old; a hallmark genetic finding in the tumors of these patients is the presence of biallelic mutations in the *RB1* gene (**Figure 9**). Retinoblastoma affects approximately 1 in 15,000 to 1 in 18,000 live births[97] and arises only from retinal cells that have lost function of

both allelic copies of *RB1*,[98] a gene located in chromosome 13q14.2 (OMIM, #180200). Unilateral Rb constitute 60% of cases and have an average age of diagnosis of 24 months,[99] while bilateral cases constitute 40% of cases and are diagnosed at an average age of 12 months.[99] Only 7-15% of all unilateral cases are capable of being transmitted to offspring[100,101] while all bilateral cases are heritable.[100] A vast majority of inherited cases present with multiple tumors in both eyes.[99] In inherited cases of Rb, a patient may carry one mutant copy of *RB1* inherited from either parent. Second hits may arise due to a separate somatic mutation, which is the case in ~30% of tumors, hypermethylation (in a small percentage of tumors), or through loss of heterozygosity at syntenic loci, including *RB1*, over a large segment of 13q (~70%).[98] The rate of loss of the second allele is high enough that >90% of patients with an inherited heterozygous mutation will develop at least one retinoblastoma.[98] The potential for mosaicism in retinoblastoma is high, as 90% of all cases have been found to be *de novo*. Only 10% are familial.[99,102] Among sporadic cases, 60% are unilateral and 40% bilateral.[99]

Trilateral retinoblastoma refers to the presence of intracranial tumors, including pinealoblastomas tumors of the suprasellar region, or tumors of other midline brain structures, in addition to either unilateral or bilateral retinoblastoma. Trilateral retinoblastoma is considered to be a syndrome of *RB1,* and is seen in 0.6-12.7% of patients with either unilateral or bilateral Rb (**Figure 9**)**.**

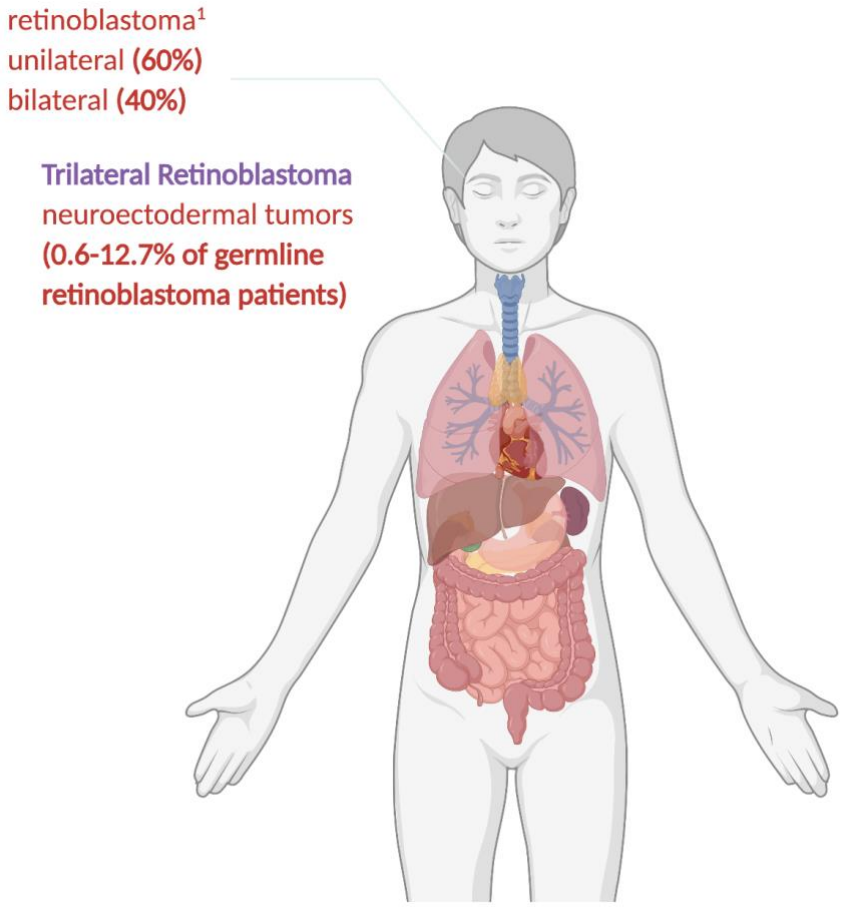

Figure 9 | Clinical features of retinoblastoma. Retinoblastoma is a malignant tumor, as indicated by red text. 1. Lohman DR and Gallie BL *GeneReviews®,* 1993 (updated November 21, 2018)

Individuals with retinoblastoma are at a higher risk of developing extraocular tumors, which are most often osteosarcomas, soft tissue sarcomas, or melanomas. For this reason, external beam radiation therapy, which increases the risk of second primary tumors to 50%, should be avoided and only used as a last resort treatment.[99] Currently, targeted therapeutics are being investigated. Nutlin-3, which blocks MDM2-mediated p53 degradation, showed

success in preclinical trials. MYCN, SKP2, E2F, and CDK inhibitors have also been recommended for future drug development investigations.[103]

Mosaic cases of the disease have been seen in around 10-30% [98,104] of affected patients, although the true incidence may be much higher, as early studies included only patients with a known family history of Rb.[98] 50% of sporadic retinoblastoma cases do not have a mutation detectable in blood using conventional Sanger sequencing.[101] More sensitive analysis using MPS is still a nascent but promising approach.

Mosaicism has been described as a major cause of low-penetrance Rb. One study identified 6 gonadal mosaics who had children with full heterozygous Rb. None of these parents developed retinoblastoma. Of the remaining 9 mosaics identified in this study, two had unilateral Rb.[98] Using MPS, analysis of retinoblastoma cells in parents of two families with affected children found VAFs of ~28% in an unaffected mother and ~34% in a father with retinoma.[104] Despite ocular tumors being the predominant feature of Rb, mosaic mutations are detectable in blood, as was demonstrated in a Singaporean study in which Sanger sequencing identified 3 mosaic variants in blood at levels of 2%, 4%, and 60%.[105] MPS has improved the rate of mutation detection from 96% to 97% in bilateral cases and from 13% to 18% in unilateral cases.[106]

Re-analysis of 40 retinoblastoma cases using MPS revealed a rate of 15% (6/40) mosaicism in sporadic disease.[101] Three of these cases had previously been determined to be heterozygous following blood analysis, but after additional MPS analysis of ocular tissue, urine, or oral mucosa, were reclassified as mosaics. The remaining three mosaic cases had NMI status before multi-tissue testing. These findings reinforce the importance of MPS and multi-tissue testing in diagnosing mosaicism.[101] A previous study which provided extensive

classification the mutational spectrum of *RB1* analyzed 529 patients from the Dutch National Retinoblastoma Register who were being treated for Rb. Of 140 non-familial cases who were found to have a germline *RB1* mutation, 14 (10%) had mosaic variants. Interestingly, 11 of the mosaic patients had bilateral Rb.[106] Another study using MPS similarly detected a greater proportion of bilateral mosaic cases (6/20) than unliteral mosaic cases (4/70).[107] Historically, studies using allele specific-PCR have also identified more mosaicism in bilateral than in unilateral patients (5.5% vs 3.8%, respectively).[108] It is likely that the number of low-level mosaic variants may still be under-detected in unilateral cases.

Germline mosaicism has been known to occur in retinoblastoma, as evidenced by four children with constitutive *RB1* variants born to the same mother but with three different, unaffected fathers. The mother was determined to harbor a germline mosaic variant.[109] A study was conducted to assess the risk of recurrence in parents with germline mosaicism. Dehainault et al. used targeted MPS to analyze blood samples from the parents of 124 sporadic Rb patients whose parents had previously tested negative for *RB1* variants with Sanger sequencing. Siblings of the affected patients were also assessed for *RB1* to estimate possibility of germline mosaicism in the parents. Only one parent was found to carry an *RB1* variant at 11% VAF in leukocytes. This corresponds to a rate of 0.8% risk of *RB1* mosaicism in a parent if their first child is affected with Rb. Additionally, there is a max risk of recurrence in a second child of 0.4%, if all of the affected parent's germline cells carry the mutation. It was determined in this study that cells of the hematopoietic lineage are not recommended as an initial tissue to sample when looking for *RB1* mosaicism.[102]

Of 1404 Rb index cases who presented to the Institut Curie for genetic consultation, 497 of 538 (92.4%) of those with bilateral Rb were germline variants, compared to only 109

of 866 (12.6%) germline variants seen among those with unilateral Rb. Germline variants more commonly underlie bilateral Rb. Additionally, there was a notable bias towards females in the non-germline PV group. 419 (54.8%) female patients had no identifiable germline variants. 346 (45.2%) had no identifiable germline variants. Males with no germline PVs were also more likely to have bilateral Rb [23 males (71.4%) vs. 12 females (34.3%).].[110] It has also been determined that paternally inherited hypomorphic variants are associated with a higher risk of recurrence.[104]

In addition to a high rate of mosaicism underlying phenotypic variability, a parent-of-origin effect has been demonstrated to affect penetrance. Maternally inherited hypomorphic pathogenic variants have been shown to retain enough *RB1* function to ameliorate the risk of tumor development.[104] Additionally, the parent-of-origin effect is known to cause of low-penetrance phenotypes in families with common and weaker inherited pathogenic variants.[104] In a separate study investigating the parent-of-origin effect, five families supported the hypothesis that maternal inheritance of common PVs is associated with a milder phenotype, zero clinical penetrance, or non-malignant retinoma development.[104] This finding carries important implications for risk of transmission. Following this model, an unaffected man born to a mother with Rb would still carry the variant, and risk of transmission to his offspring would be 50%, with no protection from the maternal parent-of-origin effect.[104] Of note, the study also found that families with segregating c.1981 C→T variants in *RB1*, extraocular second primary tumors, such as osteosarcoma and leiomyosarcoma arose in adults who had not developed retinoblastomas.[104]

TP53 – Li-Fraumeni Syndrome

TP53, on chromosome 17p13.1 (VarSome: The Human Genomic Variant Search Engine, Gene: *TP53*), is the most commonly mutated gene in hereditary cancers.[111] Germline pathogenic *TP53* variants are also the most common cause of Li-Fraumeni syndrome (LFS), a genetic disease predisposing individuals to early-onset sarcomas, brain tumors, adrenocortical carcinoma (ACC), premenopausal breast cancer, and leukemia, among other rarer tumors (**Figure 10**).[111] *TP53* contains 11 exons[105] and encodes the ubiquitous p53 protein, which acts in the nucleus and is important in transcriptional regulation, miRNA processing, cell cycle control, apoptosis, angiogenesis, iPS cell generation, and aging (OMIM, #191170). The precise rate of *TP53* mutation in LFS is still undetermined, and has been suggested to be as rare as affecting 1 in 20,000 individuals to 1 in 3,555 to 1 in 5,476 individuals.[36,112] Germline *TP53* mutations are seen in up to 75% of patients with classic LFS.[113,114] *De novo* mutations may represent at least 14% of pathogenic variants.[111,115] In general, children with germline *TP53* PVs have a 42% risk of developing a *TP53*-related cancer before the age of 16.[111] The lifetime likelihood of a male germline carrier developing cancer is about 73% in males.[111,114] The risk of developing breast cancer is almost 100% in females carriers.[113] Life expectancy for patients with LFS is severely reduced.[111]

Genetic modifiers account for phenotypic variability within and across LFS families, but there is no predictive algorithm to determine age-of-onset.[113] Definitive diagnosis of LFS requires a sarcoma diagnosed before 45 years of age, a first-degree relative with cancer onset before 45, and a first- or second-degree relative with cancer diagnosed before 45 or a sarcoma at any age.[116] The 2009 Chompret criteria outline more relaxed criteria for diagnosis of LFS. These include at least one LFS-component tumor (soft tissue sarcoma,

osteosarcoma, brain tumor, premenopausal breast cancer, ACC, bronchoalveolar lung tumor, or leukemia)[113,116] before the age of 46 and a first- or second-degree relative with any LFS tumor, excluding breast cancer, before age 56.[113] Additionally, if no family history is recorded, an individual is diagnosed with LFS if they present with at least two LFS-component tumors, again excluding breast cancer, or with ACC or choroid plexus carcinoma (CPC) at any age.[115,113,116] However, in patients meeting the Chompret criteria, *TP53* is identified in fewer than 20% of cases.[114,117]

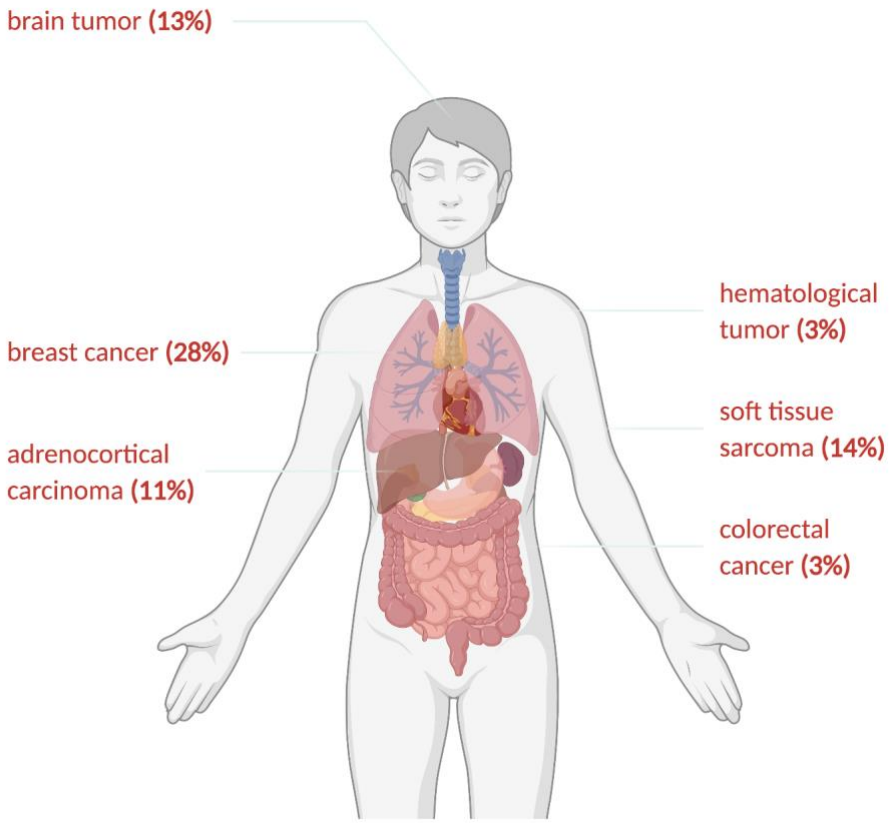

Figure 10 **| Clinical features of Li-Fraumeni syndrome.** LFS predisposes individuals to malignant tumors, indicated in red. Guha T. and Malkin D., Cold Harbor Perspectives in Medicine, 2017.

There are limited studies on LFS mosaicism.[31] Instead, much attention has been focused on the possibility of clonal hematopoiesis of indeterminate potential (CHIP) and blood-limited mutations to negative testing in other tissues.[31,111,118] In a recent study, 98 patients with apparently germline *TP53* mutations elected to have follow-up testing either by having family members undergo testing, by having their own fibroblast samples assessed, or both. They were all previously determined to have 30-70% VAF in blood and saliva

following MPS hereditary cancer genetic testing.[111] This broad range of VAFs in categorizing "likely pathogenic" germline variants was intended to account for germline allele frequency imbalances.[111] VAFs below 50% are considered mosaic, so it is likely that this cohort was already enriched in patients with somatic mutations. Nine patients who underwent only fibroblast testing only did not harbor the mutation in the samples tested.[111] Six patients who underwent both fibroblast testing did not have mutations identified in the follow-up. These 15 patients were determined to carry somatic variants. Interestingly, looking again at the preliminary MPS testing, 13/15 of this group had blood or saliva VAFs below 50%, while those who were confirmed and likely germline after follow-up had preliminary VAFs clustered around 50%. None of the somatic cases met clinical criteria for classical LFS. Additionally, the somatic cases diagnosed at a later age had VAFs between 34-57% as compared to VAFs from 30-46% in those diagnosed at an earlier age.[111] Mosaicism is certainly a possibility for the somatic group. However, because variants were absent from fibroblast samples, CHIP was proposed as an alternate mechanism of variant acquisition in blood, which would also explain the higher VAFs seen in those presenting at a more advanced age.[111] Confirming mosaicism in the 15 somatic patients would require wider tissue sampling. This study did not confirm any mosaic cases.

Another recent study explored the possibility of "blood-limited" as opposed to constitutional *TP53* PVs.[116] 145 (49.8%) of 291 patients had apparent mosaic PVs following MPS performed on blood, buccal samples, or oral rinse using a 64-gene hereditary cancer panel. Of the entire cohort, 90% were women, and 71% of the women tested had a history of breast cancer. The apparent mosaics were less likely to have breast cancer or sarcoma, and more likely to have ovarian cancer than the apparently heterozygous group. Only five of 36

apparently mosaic patients who underwent follow-up fibroblast testing had PVs at mosaic levels in fibroblasts.[116] Only one of these patients met the relaxed Chompret criteria for LFS, while the rest had breast cancer histories, but did not meet LFS diagnostic criteria.[116] Because targeted Sanger sequencing was used for follow-up analysis, it was suggested that those with negative fibroblast samples be analyzed on a case-by-case basis. Those presenting at older ages may likely have blood-limited variants, whereas a patient presenting at age 40 might more likely be mosaic and would require further testing.[116] Finally, one other previous study supported CHIP, or expansion of a hematologic neoplasia, as a phenomenon underlying a quarter of cases in which *TP53* variants were detected in multi-gene panel tests without ruling out the possibility of mosaicism.[118] Considering these three studies, MPS and standard sequencing alone are not sufficient to distinguish between the two possibilities of CHIP and mosaicism.

The largest study attempting to identify and confirm the role of mosaicism in LFS analyzed 336 patients with *TP53* PVs and reported 8 mosaic cases out of the 48 patients with *de novo* mutations. PVs were identified in blood by Sanger sequencing in two mosaic patients and by MPS re-analysis in the remaining 6 who had no mutations detected by Sanger sequencing or quantitative multiplex PCR of short fluorescent fragments (QMPSF). Availability of tumor tissue in 3 mosaic patients confirmed the role of *TP53* LOH in tumor formation.[115]

Severe mosaic LFS has been reported in two case studies.[114,117] In the first recorded case of somatic mosaicism in *TP53*, a young girl presented with adrenocortical adenoma at age 1 and osteosarcoma at age 5.[114] Both of these tumors originate from the mesoderm. In, blood samples, which also derive from the mesoderm, mutant alleles were detected in about

1/3 of lymphocytes. Interestingly, in buccal cells, which derive from the ectoderm, normal and mutant alleles were present in equal proportions.[114] Another severe mosaic case was documented in a young boy with unaffected parents who, following exome sequencing, was found to have *TP53* mutations with allele frequencies between 3.3-20% in blood and tumor tissues.[117] Presence of two soft tissue mesenchymal tumors, which derive from the mesoderm, and a metastatic neuroblastoma, derived from ectoderm, demonstrate that the mutation occurred prior to gastrulation.[117] Before validation of mosaicism in these two studies, only one case of LFS due to germline mosaic *TP53* had been suspected in an unaffected mother who had two sons with full LFS.[119] Unrelated to LFS, mosaicism in *TP53* has been reported in only one patient with bilateral choroid plexus carcinoma and in breast cancer.[120]

DISCUSSION

In recent years, the incidence of mosaicism in tumor suppressor gene syndromes has been shown to be much higher than previously thought. The advent of MPS methods along with multi-tissue sampling has made it possible to detect many previously masked and undiagnosed cases of low-level mosaicism. Better detection and clinical recognition of mosaicism is important for more precise and accurate genetic counseling and diagnostics.

Previously, Sanger sequencing was only able to detect mutant allele frequencies above 10-15%, excluding significant proportions of cases with lower allele frequencies. Now, MPS provides high depths of mean coverage between 300x and 1000x, as compared to 30-100x achievable with Sanger sequencing.[26] In TSC for example, it was once believed that mosaicism was rare in patients who were classified as no-mutation identified even following ultra-deep sequencing. However, MPS has shifted the proportion of mosaic cases detectable in TSC NMIs from 3% to 58%.[4]

Research in both TSC and NF2 has especially improved sensitivity of detection by methodically honing in on populations with a high likelihood of mosaic mutation. TSC NMI patients were re-analyzed in several subsequent studies, each providing a precise estimation of mosaicism levels using tools with higher depth of coverage.[70,64,67,4] Recently, a diagnostic algorithm, proposed by Treichel et al., suggested stratifying patients based on clinical features. Those with features common of mosaic cases should be assessed using MPS as a first line of analysis.[74] Used in conjunction with MLPA to detect copy number variants, MPS is the most promising tool to date, with 93% sensitivity in detecting *NF2* mutations in the blood and tumors of second-generation families. In this 2020 study analyzing 1055 *de novo*

NF2 cases, second-hits in tumors were only detected at a rate of 84%, pointing to the necessity of more sensitive techniques to detect mutations in tumors which are often impure and heterogeneous in cell type.[27] In TSC research, targeted MPS has a detection rate of up to 99%[73] and has identified variant allele frequencies as low as 0.77%.[70] RB1 has seen similar success in mutation detection. A combination of MLPA, direct sequencing, deletion screening, copy number gene dosage analysis and methylation assays was capable of detecting 94.9% of mutations in blood and tumors in a Singaporean cohort of 59 RB patients.[105] The extent of genetic analysis using MPS on multiple tissue types is limited in PHTS.[83,85] Larger patient series must be studied to determine a more representative range of VAFs that are common in mosaic diseases of the PHTSs. (**Table 1**)

Table 1. Summary of most recent progress in molecular analysis of TSGs and rates of mosaicism

TSG	Success rate of mutation identi-fication	Molecular technique	Frequency of mosaicism	Persistent NMIs	Tissues analyzed	Risk of transmission
NF1	(94-99%)[20]	MPS, MLPA, FISH	(1/36,000 – 1/40,000 individuals)[37]	N/A	blood, urine, skin fibro-blasts	(5%)[20]
NF2	(90-95%, blood)[49] (68%, epend-ymoma)[49]	MLPA, MPS	(25-59%)[27,59]	(5.6%)[49] (33.2%)[57] (40.6%, sporadic)[27]	blood, tumors	(5-12%)[32]
TSC1/ TSC2	(85-99%)[4,73]	MPS	(7.5-15%)[4]	(10-15%)[4]	skin lesions, blood	(2-3%, in apparently sporadic parents)[64]

PTEN		MPS	N/A	N/A	blood	N/A
CS	(85%, meeting full diagnostic criteria)[121] (25%, meeting less stringent criteria)[121]	MPS	N/A	(15%)[121]	endocervical mucosa, colonic mucosa, dysplastic gangliocytoma	N/A
BRRS	(60-70%)[121]	MPS	N/A	N/A	epidermal nevi, vascular malformations, overgrown muscle samples	N/A
VHL	(89-95%)[92]	MPS, Sanger sequencing, PCR-based deletion search	(5-8.5%)[91,92]	(5%)[90]	blood and tumors	N/A
RB1 **(bilateral)**	(92-97%)[106]	MPS, array-CGH, Sanger sequencing	(0.8%)[102] (9.3%)[122] (5.5%)[108] (10-30%)[98,101,104]	(5.1%)[105] (5.2%)[108] (7.5%, prior to multi tissue testing)[101]	blood, ocular tumors, urine, oral mucosa	(0.7-1.3%, from clinically unaffected parent)[102]
RB1 **(unilateral)**	(92.7-94.9%, tumor)[106] (13.5-42.4%, blood)[106]	MPS, QM-PCR, array-CGH, MPS	(1.3%)[98] (3.9%, tumor tested)[108] (3.7%, no tumor tested)[108] (7.3%)[105] (10-30%)[108,101,104]	(86.5%)[108]	blood, ocular tumors, urine, oral mucosa	(0.5% risk in unilateral probands who have NMI in blood)[108]
TP53	(60-80%, meeting classic LFS criteria)[112]	MPS	(2.4%)[115] (12.4%)[116]	0/336 0/221 0/291	blood, fibroblasts, ACC, breast	N/A

					sarcoma, breast carcinoma, saliva	

By contrast, no MPS studies specifically assessing rates of mosaicism have been conducted on large NF1 cohorts. In a systematic review of the literature published in 2016, Garcia-Romero et al. determined that only 15 out of 157 mosaic NF1 cases documented between 1977 and 2012 had undergone genetic testing, only 12 of which harbored a pathogenic variant.[37] This is of particular interest, as NF1 is the most common of the seven TSSs, affecting 1 in 3,000 births.[20] For comparison, NF2 is 10 times rarer, affecting 1 in 33,000 births.[32]

There is a wide range of detection rates of each TSG. This is in part due to different sampling criteria across studies. Some groups ascertained patients from national registries who meet strict diagnostic criteria and others specifically looked at patients who fall short of full diagnostic criteria but whose phenotypes are suggestive of a particular syndrome. Detection rate also depends on the tissue types sampled and analyzed. The importance of varied tissue sampling is evident in unilateral RB1, which has a major discrepancy between rate of detection in blood (as low as 13.5%)[108] and rate of detection in ocular tumor tissue (as high as 94.9%).[105] The sensitivity and specificity of genetic methods applied and robustness of computational tools used for analysis also account for detection rate variability. Older conventional sequencing methods, such as Sanger sequencing, might also have only been used to analyze the expressed exons of the genome, or the exome, in some studies.[107] Finally, relevant tumor tissues are often difficult to access and small sample sizes may confound the true rate of mosaicism in the populations reviewed here.

Mosaic TSSs Demonstrate Phenotypic Heterogeneity

As demonstrated in the literature review of eight TSGs, *NF1, NF2, TSC1, TSC2, PTEN, VHL RB1,* and *TP53,* syndromes associated with pathogenic variants bear immense phenotypic variability despite the genes' biochemical relatedness and involvement in the same pathways. Clinical features depend on the cell and tissue types affected and there is a great degree of heterogeneity within each syndrome. Considering each disease's severity and degree of malignancy, it is evident that the most lethal syndromes contain the highest rates of *de novo* mutation and mosaicism. *De novo* mutations are those which present for the first time in a patient with no known family history of a disease. These encompass somatic mutations occurring in adulthood, mosaic mutations acquired during the early postzygotic stages, as well as mutations inherited from an unaffected parent who could have low level mosaicism confined to the germline. In syndromes whose patients largely do not survive past adulthood or whose features make it unlikely that patients would be able lead normal lives, loss of the associated TSG from the cell, e.g. *NF2,* carries a higher risk of reduced reproductive fitness. Sporadic mutation allows these more deleterious gene variants to survive in the population, explaining the relatively high rate of mosaicism seen in NF2 as compared to the lower rates seen in LFS or VHL.

Mosaicism has been reported in 10-20% of *RB1* cases, with low-level germline mosaicism in sporadic bilateral RB comprising 5% of these cases.[105] Mosaic NF1 is 10-30 times less prevalent than constitutional NF1 in the general population.[45] 10-40% of patients with *de novo PTEN* mutations have confirmed mosaicism and most frequently present with a generalized distribution of cutaneous hamartomas and internal lesions resembling inherited

Cowden syndrome.[11] The true rate of *VHL* mosaicism is still undetermined, but as 5% of patients meeting diagnostic criteria have no mutation identified, mosaicism may underlie a significant proportion of these cases.[91] Mosaicism in TSC is estimated to be between 10-15%.[73] The rate of *TP53* mosaicism is still undetermined.

NF2 demonstrates the highest rate of mosaicism. 25-33% of all cases have been demonstrated to be mosaic.[27] Survival rate depends on type of mutation, with truncating mutations showing the highest mortality and with mosaic patients showing < 1/3 the mortality seen in non-mosaic, constitutional cohorts.[57] Whereas *de novo* mutations comprise half of all NF2 cases,[32] they are responsible for 90% of all RB1 cases,[102] making retinoblastoma another syndrome highly analyzed for mosaicism.

The *NF1* gene is the largest discussed in this review, containing 61 exons and covering 476.74 kb (Ensembl database, *NF1*).[123] This correlates with Type 1 neurofibromatosis' higher prevalence in the population.[20] For a comparison of relative TSG sizes: *NF2* has 17 exons (157.77 kb),[123] *RB1* contains 27 exons spanning 200 kb (928 aa)[106], *TSC1* contains 23 exons (92.17 kb),[123] *TSC2* has 41 small exons spanning 68.93 kb, *TP53* contains 11 exons (42.76 kb),[123] *PTEN* contains 9 exons (179.79 kb),[123] and the smallest, *VHL,* contains only 3 exons (19.74 kb)[123] (OMIM, #607379, 608537, 614041, 605284, 191092, 191170, 601728 respectively).

Sex-related prevalence has only been demonstrated in non-germline cases of retinoblastoma, with females exhibiting a higher rate of non-germline mutations likely acquired in the first few months of life.[110] Additionally, males with non-germline retinoblastoma showed a higher rate of bilateral Rb, a more severe clinical presentation.

These biases could be attributed to either a parent-of-origin effect causing protective, epigenetic changes in females, or to an X-linked gene modifier.[110]

While mosaicism in TSC and NF2 are known to be associated with a milder phenotype in most cases, severe mosaic disease has been reported in VHL, PHTS, LFS, and one TSC patient.[63,30,79,92,114,117] In these cases, it is possible that *de novo* pathogenic variants in other disease-causing genes could be exacerbating what would be a mild mosaic phenotype. Further studies might assess the roles of other genes in causing tumors and lesions typically associated with TSSs.

Genotype-Phenotype Correlations

Identifying mutation types within NF1 is important, as microdeletions are most often associated with severe phenotypes.[35] Additionally, because up to 60% of atypical deletions likely exhibit mosaicism,[35] more detailed analysis of atypical deletions is required.

Genotype-phenotype associations are well-established for NF2. Missense mutations have been shown to produce the least severe phenotypes and lowest mortality rates.[60] The most severe phenotypes are associated with nonsense and frameshift mutations that result in gene truncation, followed by large deletions whose phenotypic outcomes are more variable.[60] Constitutional truncating mutations were associated with the most severe phenotype along with impaired vision, hearing, and mobility along with lower reproductive fitness.[60] Splice-site mutations are associated with more severe disease states when found in exons 1-5 than in 11-15.[57]

In TSC, *TSC2* variants are associated with a more severe phenotype than *TSC1* variants. Patients with *TSC2* mutations have more severe phenotypes, seizures with an earlier

age of onset and that are more refractory and difficult to treat, and higher rates of infantile spasms, autism spectrum disorder, and intellectual disability.[9] Notably, one study showed that *TSC2* mutations in exons 23-33 have a possible lower risk of infantile spasms.[9] Due to its smaller size, *TSC1* has a lower frequency of second-hit events. *TSC1* variants are thus associated with a less severe phenotype, lower tumor counts and fewer cortical tubers.[9] Mosaic variants at VAF <10% and NMI are associated with a milder phenotype, fewer seizures and fewer cortical tubers.[9]

Genotype-phenotype investigations for Rb primarily assess the correlations between mutation types and the presence of unilateral or bilateral retinoblastoma. Germline nonsense variants are associated with an earlier age of diagnosis and higher risk of bilateral retinoblastoma, while missense, large rearrangements, and in-frame splice variants are associated with a lower risk of bilateral retinoblastoma. Somatic and mosaic variants often manifest in unilateral Rb.[110]

It is possible that specific genotypes are responsible for the different phenotypes of VHL (type 1, type 2A, type 2B, and type 2C). Truncating and missense mutations that significantly affect protein folding are associated with type 1, which is distinguished by a low risk of pheochromocytoma. Several studies have reported reduced risk of both renal cell carcinoma and pheochromocytoma in patients with a complete or partial deletion of *VHL* extending in the 5' direction to include *BRK1*. It is possible that this phenotype is associated with subset of type 1 (type 1A) VHL. Missense mutations are also associated with the type 2 phenotypes, which carry a high risk of pheochromocytoma.[90]

No concrete correlations have been established in the *PTEN* hamartoma tumor syndromes. However, in some studies, certain germline mutations were overrepresented in

patients with particular PHTS features: frameshift mutations in thyroid cancer, nonsense mutations in colorectal cancer, promoter mutations in breast cancer, and missense mutations in ASD. Mutations that result in accumulation of stable, inactive *PTEN* protein are predicted to lead to more severe PHTS developmental phenotypes and malignancies.[121]

Interestingly, there is a possible founder effect in the Brazilian population for the germline *TP53* variant, R337H, which predisposes individuals to adrenocortical carcinoma.[113] Childhood-onset ACC associated with the R337H variant has a low penetrance of 1 in 30 to 1 in 40 in carriers,[112] but the carrier rate in the Brazilian population may be as high as 1 in 350 individuals.[113] This makes LFS almost ten-times times as prevalent in Brazilians as in the general population (1 in 3,555 to 1 in 5,476).[112]

Improving Rates of Mosaicism Detection

When mosaic mutations are detectable in blood, it is possible to rule out the possibility that the variants merely represent circulating tumor DNA. This can be done if a patient is found to have a pathogenic variant in at least two primary tumors, confirming true mosaicism, or if a mutation is still detectable in blood following successful surgical resection of tumors. This has been demonstrated in mosaic Li-Fraumeni syndrome.[115] Intuitively, detection of mosaicism also relies on presence of the mutation in non-malignant tumor tissue to ensure that the mutation was acquired postzygotically rather than sporadically in adulthood. In *TP53,* mutations limited to blood may be indicative of clonal expansion rather than somatic mosaicism in adults diagnosed at an older age.[116] This calls into question whether other TSS cases with mosaicism detected only in blood are truly mosaic. Another problem encountered is lower rates of mutation detection in patients who do not meet the

diagnostic criteria for full heterozygous disease, which is often the case in mosaic patients. In LFS, for example, *TP53* mutations have only been identified in fewer than 20% of patients meeting the more relaxed Chompret criteria.[117] Better detection of mosaicism may prevent the need for costly and unnecessary screening or prevention measures.

Future directions and perspectives

One of the most important applications of knowledge about mosaicism is in providing appropriate genetic counseling. Very little is known about the risk of transmission to offspring of mosaic patients based on their VAFs and extent of mosaicism.[20,49,85] Many retrospective chart analyses have been conducted to classify the phenotypic spectrums of TSSs, but these cohorts often lack information about the genetic makeups and inheritance patterns of the patients analyzed.[37,45,96] The risks of transmission recorded for sporadic and mosaic cases are reported from what has been identified thus far in the literature and reflect current data analysis. No conclusions can yet be made about the true risk of transmission of mosaic syndromes. Additionally, the processes driving the organ-specific development of syndromic features are still poorly understood. Mosaicism is one of the mechanisms, including intronic mutations and epigenetic modifiers, that may underlie phenotypic heterogeneity within tumors and syndromes.

Currently, targeted therapeutics, such as everolimus in the treatment of TSC, show promise in reducing tumor burden in TSSs that are diagnosed early. However, drug therapeutics have not shown as much success in treating other TSSs with minimal side effects. Considering the common pathways that TSGs act within, this area of research is

extremely promising in increasing life expectancies and creating better prognoses for the syndromes discussed.

Because many of the syndromes discussed manifest with internal tumors that are difficult to biopsy, one of the biggest limitations in study design across syndromes is the limited availability of tumor tissue. One solution may be the development of standard diagnostic algorithms, as has been proposed for TSC, which allow clinicians to determine whether a patient is a likely candidate for a mosaic TSG. Regular screening may then be recommended based on mosaic clinical findings. Future research should focus on re-analyzing cohorts of patients who have had no mutation identified. It is clear that many patients with mosaic cases of hereditary disease do not carry mutations in blood at a detectable frequency using standard sequencing methods. Multi-tissue sampling has proven to uncover mosaic variant allele frequencies in both lesioned and normal tissue. With the availability of powerful tools like MPS, more sensitive detection of mosaicism coupled with better recognition of common mosaic clinical phenotypes will certainly improve understanding of low-level disease across tumor suppressor gene syndromes for clinicians and geneticists, improving the treatments, outcomes, and lives of patients with these hereditary diseases.

LIST OF JOURNAL ABBREVIATIONS

Acta Neuropathol Commun	Acta Neuropathological Communications
Am J Hum Gent	American Journal of Human Genetics
Am J Med Genet	American Journal of Medical Genetics
Am J Med Genet A	American Journal of Medical Genetics – Part A
Annu Rev Genom Hum Genet	Annual Review of Genomics and Human Genetics
Annu Rev Med	Annual Review of Medicine
BMC Med Genomics	BMC Medical Genomics
Br J Dermatol	British Journal of Dermatology
Cancer Genet	Cancer Genetics
Cancer Res	Cancer Research
Cancer Sci	Cancer Science
Cell Physiol Biochem	Cellular Physiology and Biochemistry
Childs Nerv Syst	Child's Nervous System
Clin Genet	Clinical Genetics
Cold Spring Harb Perspect Med	Cold Spring Harbor Perspectives in Medicine
Dermatol Clin	Dermatologic Clinics
Dev Med Child Neurol	Developmental Medicine and Child Neurology
Dtsch Arztebl Int	Deutsches Ärzteblatt International
Eur J Hum Genet	European Journal of Human Genetics
Eur J Med Genet	European Journal of Medical Genetics
Fam Cancer	Familial Cancer

Genes Chromosomes Cancer	Genes, Chromosomes, and Cancer
Genet Med	Genetics in Medicine
Handb Clin Neurol	Handbook of Clinical Neurology
Hum Genet	Human Genetics
Hum Mol Genet	Human Molecular Genetics
Hum Mutat	Human Mutation
Int J Mol Sci	International Journal of Molecular Sciences
JAMA Ophthalmol	JAMA: The Journal of the American Medical Association – Ophthalmology
J Cell Mol Med	Journal of Cellular and Molecular Medicine
J Child Neurol	Journal of Child Neurology
J Clin Invest	Journal of Clinical Investigation
J Cutan Med Surg	Journal of Cutaneous and Medical Surgery
J Genet	Journal of Genetics
J Invest Dermatol	Journal of Investigative Dermatology
J Med Genet	Journal of Medical Genetics
J Mol Diagn	The Journal of Molecular Diagnostics
J Pediatr Genet	Journal of Pediatric Genetics
N Engl J Med	New England Journal of Medicine
Nat Commun	Nature Communications
Nat Neurosci	Nature. Neuroscience
Nat Rev Dis Primers	Nature Reviews. Disease Primers
Nat Rev Genet	Nature Reviews. Genetics

Neurooncol Adv	Neuro-Oncology Advances
Orphanet J Rare Dis	Orphanet Journal of Rare Diseases
Pediatr Blood Cancer	Pediatric Blood and Cancer
Pediatr Dermatol	Pediatric Dermatology
Semin Pediatr Neurol	Seminars in Pediatric Neurology
Tenn Med	Tennessee Medicine
Trends Genet	Trends in Genetics

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