Clinical and Genetic Aspects of the Segmental Overgrowth Spectrum Due to Somatic Mutations in *PIK3CA*

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Segmental overgrowth disorders due to of somatic mutations in phosphatidylinositol-4,5-bisphospate 3-kinase, catalytic subunit alpha (*PIK3CA*), are designated as the *PIK3CA*-related overgrowth spectrum (PROS), named as a spectrum because of their clinical diversity.[1] Over the past 10 years, there have been a tremendous number of reports of various segmental overgrowth disorders.[2] Recently, somatic activating mutations in the phosphatidylinositol-3-kinase (PI3K)/Protein Kinase B (AKT)/mammalian target of rapamycin (mTOR)[1,3] pathway have been reported in several of these segmental overgrowth conditions. Whereas *AKT3* is expressed most strongly in the nervous system,[4] the other genes implicated in these syndromes including *AKT1*, *PIK3CA*, and mTOR have widespread expression[5] and, therefore, more widespread organ involvement. These genes function in the mTOR signaling pathway to control cell growth and metabolism.

Somatic mutations (ie, mutation in a small subset of cells within a tissue) are well known to occur in cancer and are only now being recognized with increasing frequency in hamartomatous lesions throughout the body. Mutations in *PIK3CA*, which encodes the catalytic subunit of the PI3K enzyme, have been identified in patients with a spectrum of involved organs, including the soft tissue, muscles, lymphatics, and vasculature.

Mutation detection has lead to a plethora of what were thought to be unique conditions to be classified as PROS disorders including congenital lipomatous overgrowth with vascular, epidermal, and skeletal anomalies (CLOVES) syndrome,[6] fibroadipose hyperplasia or fibroadipose vascular anomaly,[7] fibroadipose infiltrating lipomatosis,[8] hemihyperplasia multiple lipomatosis,[9] macrodactyly and muscle hemihypertrophy,[10] and skin disorders including benign lichenoid keratosis,[11] epidermal nevi, and seborrheic keratosis.[12] In addition, megalencephaly-capillary malformation (MCAP) and the related megalencephaly syndromes[13] as well as hemimegalencephaly,[3] which mainly involve the brain, can be caused by somatic mutations in *PIK3CA* and, thus, qualify as PROS. The data suggest that nearly any organ harboring somatic activating mutations in *PIK3CA* above a threshold mosaicism level can display hypertrophy leading to disease.

Luks et al[14] reported the finding of somatic mosaic *PIK3CA* mutations in most patients with isolated lymphatic malformation (LM) and disorders in which LM was a component feature, such as the CLOVES syndrome, fibroadipose vascular anomaly, and Klippel-Trénaunay syndrome (KTS). They used recently developed analytical methods, including droplet digital polymerase chain reaction (ddPCR) and single-molecule molecular inversion probes (smMIPs), as well as targeted capture and whole-exome sequencing (WES), to make these discoveries. They concluded that somatic *PIK3CA* mutations are the most common cause of isolated LMs and disorders in which LM is a component feature. In addition, they suggested that the search for causal mutations requires the sampling of affected tissues coupled with the application of advanced techniques that are capable of detecting low-level somatic mosaicism because the abundance of mutant cells in a malformed tissue can be so low as to preclude standard DNA-based molecular assessment. Theirs and other publications have led to a growing recognition of PROS and other mosaic diseases, culminating in a US National Institutes of Health sponsored workshop convened in 2013.[1] Experts agreed upon clinical diagnostic criteria for PROS disorders, as well as on future development of analytical techniques for detecting somatic *PIK3CA* mutations.

CLOVES	Congenital lipomatous overgrowth with vascular, epidermal, and skeletal anomalies
ddPCR	Droplet digital polymerase chain reaction
FFPE	Formalin-fixed and paraffin-embedded
KTS	Klippel-Trénaunay syndrome
LM	Lymphatic malformation
MCAP	Megalencephaly-capillary malformation
MIP	Molecular inversion probe
mTOR	Mammalian target of rapamycin
PCR	Polymerase chain reaction
PIK3CA	Phosphatidylinositol-4,5-bisphospate 3-kinase, catalytic subunit alpha
PI3K	Phosphatidylinositol-3-kinase
PROS	*PIK3CA*-related overgrowth spectrum
smMIP	Single-molecule molecular inversion probe
WES	Whole-exome sequencing

Clinical Diagnosis of PROS Disorders

The term PROS aims to encompass all the major unique clinical entities and characterize the continuum and overlap among the diagnoses unified by the detection of somatic

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gain-of-function mutations in the *PIK3CA* gene. These include brain disorders (eg, MCAP syndrome) and segmental body overgrowth (eg, CLOVES syndrome and fibroadipose hyperplasia). The MCAP syndrome is characterized by a large brain and abnormalities of the capillaries in the skin.[15] Cortical malformations, most distinctively polymicrogyria, digital anomalies like syndactyly and polydactyly, and variable connective tissue dysplasia can accompany the MCAP syndrome. CLOVES syndrome and fibroadipose hyperplasia are closely intertwined and might even comprise a single large spectrum of somatic overgrowth conditions; they can also be associated with a large brain and, thus, also overlap with the MCAP syndrome.[16] Compared with MCAP, CLOVES has a more distinctive growth dysregulation with congenital overgrowth of lipomatous tissues characterized as a truncal lipomatous mass. In addition, lymphatic and vascular malformations, as well as skeletal malformations like macrodactyly, prominent sandal-gap toes, and scoliosis, are frequently observed. Characteristic findings of fibroadipose hyperplasia include progressive segmental overgrowth associated with a hyperplasia of subcutaneous, visceral, and muscular fibroadipose tissue, combined with skeletal overgrowth, vascular malformations, testicular abnormalities, and polydactyly.[17] KTS is a rare congenital disorder first identified in 1900 in which blood and/or lymphatic vasculature fail to form properly.[18] Some individuals with KTS have mosaicism for a *PIK3CA* mutation, but because accompanying clinical details are not typical in those patients, it has been questioned whether they fit criteria for KTS. Luks et al[14] concentrated on patients in which LM was a component feature, such as in CLOVES and KTS; they found 5 specific hotspot mutations of *PIK3CA* accounting for ~80% of the patients, supporting the allelic nature of these conditions.

Experts at the recent National Institutes of Health-sponsored workshop suggested clinical diagnostic criteria for PROS disorders[1] (**Table I**). The development of PROS diagnostic criteria considered the presence of somatic *PIK3CA* mutation, the natural history of PROS, the characteristic of disorders derived from somatic mutations, and the historically defined clinical phenotypes. Patients may manifest the phenotype as category A with a diverse mosaic spectrum involving 2 or more of the listed features, and category B with 1 isolated and more tissue-specific features. Patients can have 1 or more findings from category A or B. If the mutation cannot be defined, then the disease is regarded as a presumptive PROS disorder.

Experts emphasized that the determination of PROS should be made cautiously, but a wider range of conditions including genitourinary abnormalities such as urinary incontinence, hydronephrosis, and hydroureter; gastrointestinal disturbances such as constipation and gastrointestinal bleeding; and neurologic dysfunctions including seizures, autism, and intellectual disability, might also lead to the consideration of genetic testing. By referencing clinical diagnostic criteria, clinicians can decide whether patients with overgrowth should be tested for *PIK3CA* mutations.

For the differential diagnosis of PROS, one should consider overlapping conditions (**Figure**), including Proteus syndrome, megalencephaly, polymicrogyria, polydactyly, hydrocephalus, and hemimegalencephaly, which are linked with *AKT* and *PIK3CA* mutations, as well as phosphatase and tensin homolog-related overgrowth spectrum disorders like Bannayan-Riley-Ruvalcaba and Cowden syndromes.[19,20] However, most have features distinguishing each from PROS. For example, the clinical progression of Proteus syndrome, where somatic mutations in *AKT1* were defined in affected tissues,[7] has postnatal onset overgrowth that is contrary to that observed in CLOVES and fibroadipose hyperplasia. Furthermore, in Proteus, one does not observe the typical truncal fatty-vascular mass, spinal paraspinal fast-flow lesions, and acral abnormalities, which are characteristics of CLOVES.[19]

Table I. Clinical diagnostic criteria for PROS disorders

Required criteria
1. The presence of somatic *PIK3CA* mutation (if the mutation cannot be defined, then the disease is regarded as a presumptive PROS disorder)
2. Congenital or early childhood onset
3. Sporadic, without family history and mosaic distribution
4. Affected patients can have one or more findings from category A or B.

Category A (more than 2 features)*	Category B (isolated features)
1. Adipose, muscle, nerve, and skeletal overgrowth	1. Large, isolated LM
2. Capillary, venous, arteriovenous, and/or LMs	2. Isolated macrodactyly,† overgrown and splayed feet/hands, or overgrown limbs
3. Epidermal nevus	3. Truncal adipose overgrowth
	4. Hemi or bilateral dysplastic megalencephaly or focal cortical dysplasia type 2
	5. Epidermal nevus
	6. Seborrheic keratosis
	7. Benign lichenoid keratoses

*Typically progressive. Can manifest as scoliosis (kyphosis), limb overgrowth, central nervous system (hydrocephalus, cerebellar tonsillar ectopia, Chiari, megalencephaly, mega corpus callosum), regional lipomatous undergrowth with overgrowth, infiltrating lipomatosis, Wilms tumor/ovarian cystadenoma.
†Other terms: macrodystrophia lipomatosa, macrodactylia fibrolipomatosis, and gigantism.

Somatic Mutation Detection

Somatic mutations are not inherited from the parents, but instead can arise in any of the cells of the body after fertilization and zygote formation (**Table II**). Because they do not exist in the germ cells or fertilized zygote they are considered to be postzygotic and are de novo by definition (ie, not transmitted from either parent). These mutations are present only in a portion of patient cells, typically in a restricted organ or regional distribution, the limits of which are probably determined by timing of the origin of the mutation and the migrations and proliferation developmental patterns of the originating mutant cell.[21,22]

It has been reported that tissues from biopsies of some patients with PROS have mosaicism at <5%.[1] Therefore, for diagnostic genetic testing, one should consider not only the types of mutations, but also the burden and distribution of

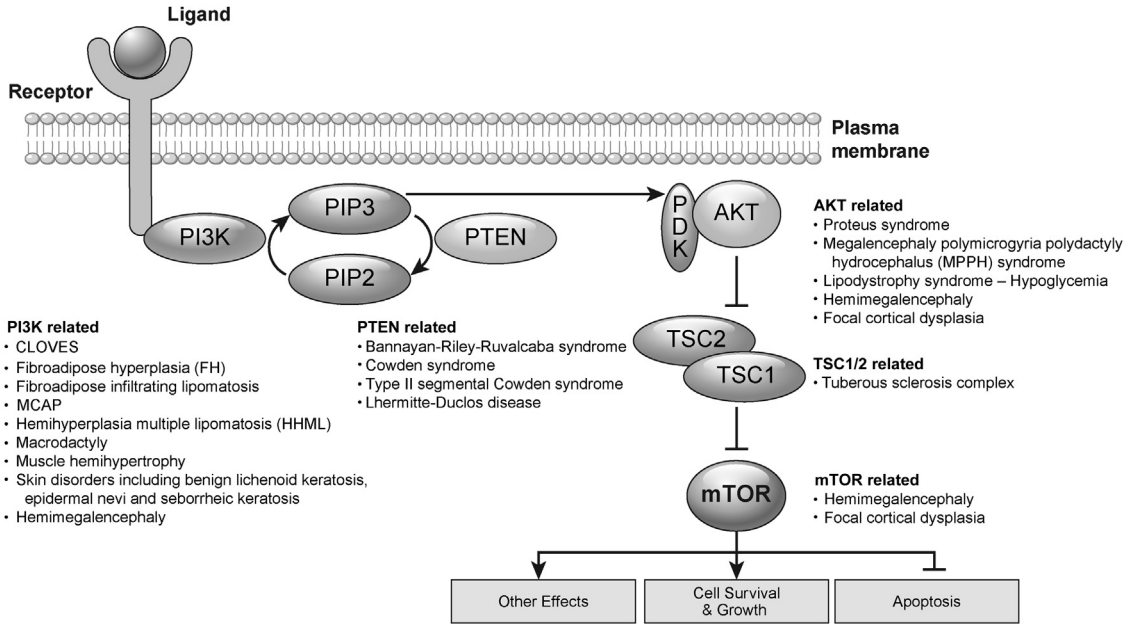

Figure. Simplified PI3K-AKT-mTOR pathway and associated clinical overgrowth disorders. *PIP*, phosphatidylinositol polyphosphate; *PTEN*, phosphatase and tensin homolog; *PDK*, phosphatidylinositol-dependent kinase.

cells that carry the mutation. To increase the detection rate, genetic testing to confirm PROS should be performed ideally using clinically affected tissue specimens, preferably those freshly obtained from an affected area or from the overgrown tissue. The detection of mutations in formalin-fixed and paraffin-embedded (FFPE) tissues can be challenging due to extensive DNA degradation, resulting in higher false-negative and failed assay rates. Recent advances in molecular detectors, such as molecular inversion probes (MIPs), post-processing of extracted DNA by treatment with uracil-DNA glycosylase, as well as the application of advanced bioinformatics tools, have allowed increased use of FFPE tissues for the detection of somatic mutations.[23-26]

Diagnostic genetic testing to identify somatic mutations for PROS is particularly challenging especially as the level of mosaicism approaches the detection limit of the technology. Conventional and clinically applicable Sanger sequencing can be used for the detection of mutations with relatively high allelic frequencies (ie, >20% mosaicism).

Recently, several molecular analytic tools for detection of *PIK3CA* and other somatic mutations have been developed with the potential to increase sensitivity and specificity, but each with its own strength and weakness.[14]

The ddPCR technology is particularly useful for screening of hotspot known mutation sites, offering superb resolution to define low rate mosaic mutations (ie, <1:100 cells with a mutation) but only for known mutant alleles.

ddPCR uses water-oil emulsion droplet technology.[27] Unlike conventional polymerase chain reaction (PCR), a single ddPCR run generates upward of ~20 000 data points, each gathered from an individual droplet and, thus, allowing for comparison of absolute counts of target DNA copies compared with the control input sample. As noted by Luks et al,[14] ddPCR is particularly useful for screening hotspot mutations, where they found 1 of the 5 hotspot mutations in the affected tissue samples from most patients with LM, CLOVES syndrome, KTS, and fibroadipose vascular anomaly. The shortcoming for diagnosis is that individual probes must be designed for each mutation to be screened and, thus, will miss all but the known alleles being assessed. Thus, ddPCR might provide an efficient and cost-effective strategy for the screening of common mutations, with superb sensitivity and specificity.

Next generation sequencing-based tools, including MIPs and WES, can detect mosaic mutations, both known alleles and novel alleles, but at a greater cost and lower sensitivity

Table II. Analytical methods for genetic diagnosis for testing of somatic *PIK3CA* mutations by using clinically affected tissue specimens

Methods	Strengths	Weaknesses	Examples of applications
Sanger sequencing	Easy accessibility	Low resolution	Initial screening of somatic mutations in "frequently mutated genes"
ddPCR	Superb resolution	Mutation-specific	Detection of somatic hotspot mutations
smMIPs sequencing	High specificity, scalability	Limited uniformity	Large-scale multiplex sequencing of candidate genes
WES	Unbiased approach	High cost	Comprehensive mutation discovery

and specificity compared with ddPCR. They also have scalability for high-throughput, multiplexed analyses to test multiple tissues from many patients simultaneously. By adjusting sequencing depth to 200- to 500-fold base coverage (which is greater than the 30- to 100-fold base coverage used for traditional WES), WES can detect mosaicism as low as about 10% of cells (ie, 1:10 cells with a mutation).[28] WES enables clinicians to discover novel, disease-associated genes and novel mutations that might be different from the common ones or others being assessed. MIPs technology, in which target sequence from candidate genes is barcoded for sample number and DNA strand number, theoretically provides single-cell sequencing resolution. By using very high sequencing depth (ie, 1000- to over 10 000-fold base coverage), MIPs have a sensitivity to detect mosaicism as low as about 1% of cells (ie 1:100 cells with a mutation).[29] Furthermore, because DNA is replicated rather than amplified, amplification errors that can occur in PCR-based assays are minimized. MIPs are also scalable for high-throughput and multiplexed analyses, in which thousands of target loci or exons can be assayed simultaneously. Using an adaptation of MIPs termed smMIP sequencing,[29] Luks et al[14] also identified disease-causing *PIK3CA* mutations in an additional 8% of the samples, in which they could not find mutations with their initial screening methodology. The downside is that smMIP does not achieve sequencing of the whole exome and, thus, the power to detect mutations in novel genes is diminished. PCR-based amplicon techniques, such as the TruSeq Custom Amplicon kit from Illumina (San Diego, California), allow researchers to generate a customized library that contains target regions each spanning hundreds of base pairs.[30] However, despite recent advances in sequencing, the PCR-based methods still have limitations because of the relatively high false-error rate attributable to amplification-induced base substitution errors, especially important for the detection of low-level mosaicism. Target enrichment by smMIPs or PCR-based amplicons techniques are also suitable for fixed samples (ie, FFPE samples), as it has been applied successfully with fragmented DNA samples.[31]

Even after applying WES and smMIPs, however, Luks et al[14] did not find candidate mutations in all patients. One possible explanation is that *PIK3CA* is not responsible for all of the LMs and other vascular malformations seen in patients with the CLOVES syndrome and KTS. Alternatively, mosaicism could be present but below the detection limit of the methods used. For example, recent publications used deep coverage (412-668×) WES followed by ultra-deep (100 000-347 499×) targeted amplicon sequencing to spot somatic mutations in the brain tissues of patients with focal cortical dysplasia with as little as 1% mosaicism (ie, 1% of cells with a mutation).[28,32] One problem is that such sensitive methods of detection have the potential for false positive results, so that the percentage of these variants that can be verified with alternative methods is still mostly unknown. Thus, physicians must consider not only the likely gene(s), the specific genetic variant, and the level of mosaicism that can cause the clinical presentation, but also the false-positive and false-negative detection rates when deciding diagnostic approaches and interpretation of genetic testing results.

Although many concerns regarding technical limitations, interpretation of results, insurance coverage, and cost remain to be answered still, these advanced molecular assays are undoubtedly going to enter into clinical practice in the near future.

Management and Genetic Counseling

An integrated multidisciplinary approach to manage and monitor PROS disorders is critical in clinical practice. First, after the presumptive diagnosis of PROS, a thorough clinical history, physical examination, cardiovascular evaluation, and baseline imaging studies, including the overgrowing tissues or organs is required to anticipate the patient's specific needs and potential future complications. Referral to the appropriate specialists is suggested based on the manifestations, such as referral to a child neurologist or rehabilitation physician for seizures, intellectual disability, behavior disorder, or speech, motor difficulties, or referral to pediatric subspecialists for cardiac problems, renal abnormalities, swallowing or feeding difficulties, and surgical or orthopedic referral for significant segmental overgrowth.[33] In addition, for MCAP syndrome, neurosurgical referral should be considered for patients showing evidence of raised intracranial pressure derived from obstructive hydrocephalus or related anomalies.[34] In CLOVES, surgical excision is sometimes required, when truncal lipomatous masses infiltrate surrounding tissues. Moreover, the risk for central thromboembolism may require active or prophylactic medical intervention.[35] Furthermore, careful monitoring is also recommended to evaluate the commonly occurring postoperative sequelae of surgical excision. For example, after the surgical excision of the truncal lipomatous mass, recurrence, hypervascularity, and infiltrative growth are possible complications so patients should be closely followed postoperatively.

Along with conservative treatments, several small-molecule inhibitors of the PI3K pathway have been characterized, some of which are currently in clinical trials as therapeutic agents for cancer treatment. Obviously, a PI3K inhibitor may help slow or even reverse symptoms in patients if drugs can silence the effect of the mutation. Preclinical data for such therapeutic strategies for PROS disorders is still lacking but is anticipated.[36] Currently, commercial testing for somatic *PIK3CA* mutations is not available, but individual hospitals or commercial entities may soon offer clinical laboratory improvement amendments-approved testing as part of a cancer panel.

For PROS, genetic counseling is relatively straightforward because the risk of recurrence is probably zero or close to zero, as the mutation is probably no more likely to occur postzygotically in a subsequent pregnancy that it was in the index case. Vertical transmission from an affected individual

to a child can probably be excluded for PROS disorders because even if the mutant cells seeded the germline and the affected individual were to conceive, a zygote with a germline PROS-associated mutation probably would not survive. Remarkably, in a small number of patients with MCAP syndrome, the disorder was derived from a de novo germline mutation in *PIK3CA* or *AKT3* (ie, the patients were not mosaic but rather carried a germline *PIK3CA* mutation derived from the germline of one of the parents).[13,37] Therefore, the recurrence risk could be substantial for subsequent pregnancies if the mutation exists in the germline of one of the parents. If such an affected or unaffected member were to conceive a child, there would be up to a 50% risk of the conception carrying the mutation, so caution is warranted during genetic counseling, especially until the field has a sense of which mutations can be transmitted and which are unlikely to be transmitted.

Conclusions

The recognition of the new entity called PROS, along with the potential for genetic testing, now allows physicians to recognize and confirm diagnosis of the condition and to anticipate better the potential clinical problems. Furthermore, recent advances in sequencing and analytic methods for genetic diagnosis of PROS enable the systematic measurement of somatic mosaicism levels in patients with PROS disorders. Because the key to treating disease lies in understanding the underlying molecular causes, it can be anticipated that targeted treatments for patients with PROS disorders will be a goal of the near future. ■

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