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Prenatal Diagnosis of CLOVES Syndrome Confirmed by Detection of a Mosaic *PIK3CA* Mutation in Cultured Amniocytes

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Abstract

Congenital lipomatous asymmetric overgrowth of the trunk, lymphatic, capillary, venous, and combined-type vascular malformations, epidermal nevi, skeletal and spinal anomalies (CLOVES) syndrome, a segmental overgrowth syndrome, is caused by post zygotic somatic mutations in *PIK3CA*, a gene involved in the receptor tyrosine kinase phosphatidylinositol 3-kinase (PI3)-AKT growth-signaling pathway. Prenatal ultrasound findings of lymphovascular malformations, segmental overgrowth and skeletal defects can raise suspicion for CLOVES syndrome, but molecular confirmation of *PIK3CA* mutations on prenatally obtained samples is challenging because of somatic mosaicism. We detected a mosaic disease-causing mutation in *PIK3CA* by sequencing of DNA extracted from cultured amniotic cells, but not from DNA directly prepared from an amniotic fluid sample in a fetus with prenatally suspected CLOVES syndrome. The infant was born prematurely and displayed severe lymphovascular malformations and segmental overgrowth consistent with a clinical diagnosis of CLOVES syndrome; he passed away at 29 days of life. We discuss the complexities and limitations of genetic testing for somatic mosaic mutations in the prenatal period and highlight the potential need for multiple approaches to arrive at a molecular diagnosis.

Keywords

somatic overgrowth; prenatal diagnosis; mosaicism; vascular anomalies; lipomatous malformation

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INTRODUCTION

Somatic overgrowth disorders are clinically and molecularly heterogeneous [Biesecker and Sapp, 2012; Mirzaa et al., 2013]. One of the more recently described segmental overgrowth syndromes is Congenital Lipomatous asymmetric Overgrowth of the trunk, lymphatic, capillary, venous and combined Vascular anomalies, Epidermal nevi, Skeletal and spinal anomalies (CLOVES) syndrome [Sapp et al., 2007; Alomari, 2009; Mirzaa et al., 2013]. The vascular and lipomatous abnormalities in CLOVES syndrome are segmental and asymmetric, typically involving the skin and underlying tissues of the chest or abdominal wall. Other characteristics include segmental epidermal nevi and asymmetric overgrowth of extremities with broad feet and asymmetrically enlarged toes. [Sapp et al., 2007; Alomari, 2009].

Kurek et al. [2012] identified somatic mosaic missense mutations in *PIK3CA* in affected tissues of six patients with CLOVES syndrome. This gene encodes a catalytic subunit of PI3K that phosphorylates AKT, activating the PI3K-AKT-mTOR growth-signaling pathway [Kurek et al., 2012]. Activating germline mutations in this pathway can lead to multiple cancer types, whereas activating somatic mosaic mutations in *PIK3CA* are associated with segmental overgrowth syndromes of the body (CLOVES syndrome and fibroadipose hyperplasia syndrome) [Kurek et al., 2012; Lindhurst et al., 2012] or the brain (Megalencephaly Capillary Malformation (MCAP) syndrome and hemimegalencephaly [Gucev et al., 2008; Riviere et al., 2012]. The CLOVES syndrome presents prenatally and its clinical features have been observed on prenatal ultrasound and fetal magnetic resonance imaging (MRI) [Sapp et al., 2007; Alomari, 2009; Alomari, 2011]. We attempted genetic testing on amniocytes from a patient with prenatally suspected fetal CLOVES syndrome, reasoning that amniotic fluid may contain cells originating from the fetal skin and peripheral vascular tissues that harbor the mosaic mutation.

CLINICAL REPORT

A G2P0 pregnant mother was referred at 26 weeks gestation to our fetal center because of multiple anomalies detected by prenatal ultrasound. A diagnostic amniocentesis performed prior to referral showed a normal male karyotype and normal chromosomal microarray analysis. Repeat imaging by ultrasound and fetal MRI was significant for multifocal cystic infiltrations of the trunk, thought to be vascular or lymphatic malformations, and asymmetric overgrowth of the left leg and foot (Fig. 1A and 1B). No other fetal abnormalities were detected, but significant polyhydramnios was noted. Upon multidisciplinary review of the fetal MRI and ultrasound images by a team of maternal-fetal medicine specialists, neonatologists, fetal surgeons, geneticists and other pediatric subspecialists, the possibility of CLOVES syndrome was raised based on similarities of the appearance of the fetal trunk and left foot (Fig. 1B) to images from previous descriptions of this syndrome [Alomari, 2009].

The mother underwent a therapeutic amnioreduction and fluid was saved for genetic testing. Mutation analysis on genomic DNA directly extracted from this amniotic fluid did not detect any known mutations in *PIK3CA*. Because *PIK3CA* mutations can be mosaic, we also

analyzed DNA extracted from cultured amniocytes of this sample and from an earlier amniocyte culture obtained from the laboratory where the initial karyotype analysis was performed. We detected 38% mosaicism for a c.1624G>A (p.G542K) mutation in *PIK3CA* (Fig. 2) in both cultures, which has been previously reported as causative for CLOVES syndrome [Kurek et al., 2012]. The infant was delivered by cesarean at 31 weeks of gestation because of non-reassuring fetal heart tracing, worsening biophysical profile and evidence of cutaneous edema in addition to fetal ascites, which was removed under ultrasound guidance prior to delivery to reduce the abdominal circumference. The mutation analysis results were available on the day of birth and helped guide medical management in the neonatal intensive care unit.

The infant's birth weight was 3.475 kg (>97th centile), length 29.5 cm (~10th centile) and head circumference 29.5 cm (~50th centile). Physical exam showed asymmetric growth of the chest and abdomen with bilateral multicystic malformations that were externally visible (Fig. 1C), but proportionate appearing head and hands. There were bilateral linear epidermal nevi, and asymmetric growth of the left leg with macrodactyly of the left foot and a sandal gap between the first and second toes (Fig. 1D). Postnatal imaging performed on day of life number five showed bilateral multicystic venous lymphatic malformations, chest wall venous ectasias, multiple congenital hemangiomas, ascites, and pleural effusions. There were no discrete lipomatous masses but nearly all of the subcutaneous fat in the trunk appeared abnormal secondary to infiltration by these lymphovascular malformations. The neonatal clinical course was complicated by pulmonary hypoplasia related to prematurity and complications related to the underlying lymphovascular malformations associated with CLOVES syndrome. The infant had persistently low hemoglobin, platelets and coagulation factors, with hemorrhage and hypotension that was unresponsive to multiple blood product transfusions. An attempt at sclerotherapy for treatment of the chest wall vascular anomalies was complicated by poor wound healing. He passed away at 29 days of age from presumed sepsis and respiratory insufficiency. The *PIK3CA* mutation was not detected in DNA extracted from peripheral blood leukocytes and no biopsies on affected tissues for further testing could be done.

METHODS

Ten ng of DNA extracted using a DNeasy DNA blood & tissue kit (Qiagen) was used for multiplex PCR amplification with the version2 Cancer Gene Mutation Panel primer mix that covers six *AKT* and 97 *PIK3CA* mutations using Ion AmpliSeq™ Kit 2.0 reagents. Amplicons were ligated to Ion-compatible adaptors after removal of amplicon-specific primers and amplified using Platinum® PCR SuperMix High Fidelity. Sequencing templates were prepared by emulsion PCR using the Ion template preparation kit with the Ion OneTouch™ 200 template system. Next generation sequencing was run on the Ion Torrent Personal Genome Machine (PGM). Results were analyzed with the Torrent Suite software and coverage analysis and variant calling were performed using the AmpliseqCancerPanel plug-in 3.4 (all reagents were from Life Technologies). Variants were then annotated using home-brew software. Positive *PIK3CA* calls were reviewed using IGV 2.2 (Broad Institute) and confirmed by Sanger sequencing. This report describes clinical work that is not considered research at our institution.

DISCUSSION

To our knowledge, this is the first description of a molecular diagnosis for CLOVES syndrome performed on DNA from amniotic fluid. Swarr et al. [2013] recently reported a diagnosis of MCAP that presented prenatally with pleural effusions and fetal hydrops, but for that patient, a molecular diagnosis was made after birth from buccal cells and cultured fibroblasts. Prenatal imaging by high-resolution ultrasound complemented with fetal MRI has improved characterization of these syndromes *in utero* but their distinguishing features can be difficult to recognize. Hence, obtaining a molecular diagnosis prenatally should help planning the perinatal management of affected pregnancies. The fact that mutation analysis on DNA from cultured amniocytes from two separate cultures, but not on DNA extracted directly from amniotic fluid, detected the causative mosaic mutation in *PIK3CA*, highlights the complexity of prenatal diagnosis of mosaic mutations and the potential need for multiple sources of DNA to achieve a diagnosis. It further shows the benefit of a multidisciplinary team to aid selection of the most appropriate genetic test and samples to be analyzed for this patient. Considering that somatic mutations in *PIK3CA* result in an overgrowth phenotype and germline mutations predispose to cancer [Mirzaa et al., 2013], we speculate that proliferative advantage of cells carrying mutations in this gene explains why only the cultures had the causative mutation.

Mosaicism has become more recognized as a mutational mechanism for human developmental disorders and birth defects and is not unique to overgrowth syndromes. It has also been observed for other disorders, such as in the rare surviving boys and some of the girls with X-linked dominant male-lethal syndromes like Goltz syndrome, caused by mutations in *PORCN* [Grzeschik et al., 2007; Wang et al., 2007], and incontinentia pigmenti, caused by mutations affecting *NEMO* [Smahi et al., 2000; Kenwrick et al., 2001]. Somatic mosaicism should be considered if patients present prenatally with sonographically detected birth defects that raise suspicion for these conditions. The presence of increased skewing of X-inactivation in affected females with a heterozygous mutation suggests that cells carrying mutations in these genes have a proliferative disadvantage and amniocyte cultures may not identify a mosaic mutation. Proliferative disadvantage may also be seen when there are mosaic marker chromosomes, for example in tetrasomy 12p (Pallister-Killian syndrome) due to the presence of a mosaic isochromosome 12p i(12p) marker, and one of the conditions that should be considered when there is a fetal congenital diaphragmatic hernia [Wilkens et al., 2012].

Thus, the source of DNA most likely to yield a result varies by the nature of the genetic mutation, the affected gene and functional consequences of the mutation, the level of mosaicism, and the affected tissues and organs involved. Multiple sources of DNA can be considered for prenatal diagnosis of genetic conditions with mosaicism. If the mutation is also present in the placenta, it can be detected in chorionic villus samples, but if there is placental mosaicism, it may remain undetected if only non-affected villi are sampled. Conversely, if there is confined placental mosaicism, a mutation could be detected that is limited to the placenta but does not affect the fetus and should be interpreted in view of the clinical presentation and prenatal imaging findings. Another option would be to analyze non-invasively sampled cell-free fetal DNA from maternal plasma, which originates from

the trophoblast and is thought to represent the entire placenta, potentially allowing more sensitive detection of a mosaic mutation present in the placenta. Interestingly, there has been a report of placental chorioangioma associated with CLOVES syndrome in a fetus [Alomari, 2009]. Although fetal tissue sampling could be considered for prenatal diagnosis of mosaic disorders, this requires a highly invasive procedure. When vascular malformations are present, such as in CLOVES syndrome, the benefits of such procedures are unlikely to outweigh the risks. When indicated prenatal procedures such as amnioreduction or removal of fetal ascites are performed, these fluids can also be submitted for genetic testing.

Current treatments for the overgrowth syndromes like CLOVES syndrome, include surgical debulking and sclerotherapy. Unfortunately, in addition to being premature, the patient was very severely affected with large lesions and had a complicated neonatal course with presumed sepsis, anemia, thrombocytopenia, and persistently low levels of coagulation factors with hemorrhages that were refractive to treatment with transfusions, precluding surgical debulking as an option. Attempts at sclerotherapy were complicated by poor wound healing. As molecular diagnosis and characterization of the phenotype and natural history of CLOVES syndrome continue to evolve, improved prenatal detection and early molecular confirmation may in the future open up avenues for targeted therapeutics directed at correcting or addressing the consequences of the underlying molecular defect [Iacobas et al., 2011].

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Fig. 1.

Prenatal images at 27 weeks gestation and postnatal images of the infant after birth at 31 weeks. A: T2-weighted coronal MR image of the fetus at 27 weeks. There is extensive infiltration by the malformations through the subcutaneous tissues of the abdominal wall, from the axillae to the pelvis, including the dorsal upper thigh on the left. Macrocystic structures are noted (arrows), and there is large-volume ascites. B: Maximum Intensity Projection (MIP) T2-weighted MR image of the limb overgrowth affecting the left foot, with wide-spaced macrodactyly. Multiple sequences showed enlargement of the entire left lower

extremity compared with the right, though without abnormal high signal that would indicate infiltration by a mass in the lower leg. C: One day-old infant with bilateral asymmetric overgrowth of the chest and abdomen, bilateral linear epidermal nevi. D: Asymmetric overgrowth of left lower limb, showing macrodactyly, furrowed foot sole and a sandal gap between first and second toes.

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Fig. 2.
The *PIK3CA* c.1624G>A (p.E542K) mutation detected in this patient using Next Generation Sequencing. The data are visualized in the Integrative Genomics Viewer (IGV)(top) and confirmed by Sanger sequencing (bottom).