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Identification and characterisation of a novel constitutional *PIK3CA* mutation in a child lacking the typical segmental overgrowth of “*PIK3CA*-Related Overgrowth Spectrum” (PROS)

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Abstract

Activating somatic *PIK3CA* mutations underlie a growing heterogeneous spectrum of segmental overgrowth disorders. We report the identification and evaluation of a novel *de novo* constitutional *PIK3CA* mutation (NM_006218.2:c.335T>A, p.Ile112Asn) in a child with congenital megalencephaly and macrosomia. Functional characterization of patient cells using a variety of endpoints demonstrates increased Phosphatidylinositol-3-kinase (PI3K) activity. The mutation lies in a linker region adjacent to the p85 (*PIK3R2*) binding domain of the p110 α (*PIK3CA*) catalytic subunit of PI3K. We show that altered stoichiometry within the p85-p110 complex likely underlies the hyperactive PI3K-AKT-mTOR signaling in this instance. Our findings expand upon the recently proposed “*PIK3CA*-Related Overgrowth Spectrum” (PROS) associated with *PIK3CA*-mutations and PI3K hyper-activation, adding constitutional *PIK3CA* mutations as an underlying cause of megalencephaly and macrosomia in newborns.

Keywords

PIK3CA; p110 α ; macrocephaly; overgrowth

PIK3CA (MIM# 171834) encodes p110 α , the catalytic subunit of class IA PI3K, and is a lipid kinase of the PI3K-AKT-mTOR network. PI3K activates AKT upon stimulation of cell surface receptor tyrosine kinases by extracellular signals. Both AKT and PI3-kinases play

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The mutation has been submitted to the ClinVar database (<http://www.ncbi.nlm.nih.gov/clinvar/>).

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critical roles in cell growth and proliferation and their constitutive activation is one of the most common mechanisms involved in oncogenesis. Somatic mutations in *PIK3CA* are found in multiple solid tumors. Moreover, mosaic mutations in *PIK3CA* cause the megalencephaly-capillary malformation syndrome (MCAP) (Riviere et al. 2012, Mirzaa et al. 2013). Indeed, hyper-activating *PIK3CA* mutations have now been described in a wide range of clinical entities including Fibroadipose Infiltrating Lipomatosis, Hemihyperplasia Multiple Lipomatosis (HHML), Dysplastic Megalencephaly (DMEG), Macroductyly and Congenital Lipomatous Overgrowth, Vascular Malformations, Epidermal Nevi, Scoliosis/Skeletal and Spinal (CLOVES) syndrome (Kurek et al. 2012, Lee et al. 2012, Lindhurst et al. 2012). To better formulate effective genetic diagnostic and management strategies, it was recently proposed to group these overlapping clinical diagnoses together as PROS; “*PIK3CA*-Related Overgrowth Spectrum” (Mirzaa et al. 1993, Keppler-Noreuil et al. 2015). In addition, several individuals with “Cowden-like” disorder were reported to have germline mutations of *PIK3CA* (Orloff et al. 2013). However, since limited clinical data were provided in the latter report regarding the phenotype and functional characteristics, with no information regarding tissues tested or results of parental testing, detailed comparisons here are difficult. Cowden syndrome has traditionally only been associated with mutations of *PTEN*, which encodes an antagonist of PI3K. Since *PTEN* mutations also cause macrocephaly and developmental delay in children, we screened a cohort of 12 children with macrocephaly and developmental delay/intellectual disability who tested negative for *PTEN* mutations, and who also had normal chromosomal microarrays.

We report a 6-year-old boy who presented with mild macrosomia (birth weight +2.44 SD, body length +1.8 SD) and macrocephaly (+3.3 SD) at birth, mild developmental delay, and minor brain anomalies (Figure 1A; Supp. Clinical Report). His weight normalized under a calorie restrictive diet during the first year of life. The macrocephaly persisted with OFC of +3.1 SD at the age of 6 years. Sanger sequencing revealed a novel *de novo* heterozygous missense variant in *PIK3CA* (NM_006218.2:c.335T>A, p.Ile112Asn). This mutation was identified in all three tissues examined (peripheral blood, buccal swab and skin fibroblasts) with no evidence for somatic mosaicism (Supp. Figure S1). This mutation is not present in available public databases (Exome Variant Server, ExAC Browser) and has not previously been reported as a pathogenic mutation. This mutation is listed in COSMIC in a single endometrial carcinoma sample at a somatic mutation frequency 0.037% (COSM24711). Another amino acid change at the same residue (p.Ile112Val) was also reported in one carcinoma sample (COSM1420752). Therefore, we set out to characterize the impact of this mutation on the PI3K-AKT-mTOR pathway.

This mutation did not affect p110 α expression in patient LCLs (Figure 2A). To assess the functional impact of the novel p.Ile112Asn mutation, we initially examined PI3K activity by indirect immunofluorescence in patient-derived primary fibroblasts by staining for phosphoinositol-3,4,5-triphosphate (PIP3), the product of PI3K. We found elevated endogenous levels of PIP3 staining specifically in patient compared to wild-type (WT) fibroblasts (Fig 2B and Supp. Figure S2). We observed a similar result using patient-derived LCLs (Supp. Figure S3). Dot blot analysis of acidic lipids extracted from LCLs also indicated elevated PIP3 in patient cells relative to WT (Figure 2C). Collectively, these

analyses are consistent with hyperactive PI3K in the patient cells. We previously reported that *PIK3CA*-mutated MCAP patient LCLs exhibit elevated PIP3 levels and elevated PI3K-AKT-mTOR signaling; the latter indicated by elevated phosphorylation of the S6-Kinase substrate S6/rpS6 (Riviere et al 2012). Here, we observed spontaneously elevated levels of phospho-S6 (pSer240/2446) upon serum starvation (NS; no serum) of patient primary fibroblasts, when compared to WT (Figure 2D and 2E). Furthermore, acute stimulation of these cells with Epidermal Growth Factor (EGF) also yielded elevated phospho-S6 reflective of hyperactive PI3K-AKT-mTOR activity, specifically in the patient fibroblasts (Figure 2D and 2E). Wild-type (WT) LCLs treated with increasing amounts of the dual PI3K-mTOR inhibitor PI-103 showed a marked progressive reduction in phospho-S6, relative to patient cells (Figure 2F). This relative insensitivity of the patient LCLs to a dual PI3K-mTOR inhibitor is consistent with spontaneously elevated PI3K-AKT-mTOR signaling in these cells. We previously reported a similar response to PI-103 in MCAP patient LCLs (Riviere et al. 2012). Further, and consistent with hyperactive PI3K-AKT-mTOR activity, we also observed elevated basal phospho-AKT (pSer473) in serum starved and EGF-treated patient primary fibroblasts, compared to WT cells (Figure 2G).

The p.Ile112Asn change lies in a short linker region between the p110 α -Adapter Binding Domain (ABD) and the p110 α -RAS binding Domain (RBD) (Figure 1E and Supp. Figure S4A). The p110 α -ABD serves as a p85 α interaction interface mediating binding to the iSH2 domain of p85 α . We hypothesized that altered stoichiometry within the p85-p110 α complex could underlie the hyperactive PI3K-AKT-mTOR signaling in this instance, likely reducing the inhibitory effect of p85 α upon p110 α . p85 α is a constitutively interacting negative regulator of p110 α . Indeed, heterozygous deletion of *Pik3r1* (p85 α) in mice leads to up-regulation of PIP3 levels and Akt activation (Mauvais-Jarvis et al. 2002). Furthermore, the iSH2 domain of *PIK3R1* is a mutational hotspot in endometrial cancer associated with increased AKT phosphorylation. Therefore, if p.Ile112Asn impacts upon the integrity of p110 α -ABD, it may weaken its' ability to interact with the corresponding iSH2 domain of p85 α , likely diminishing the negative regulatory influence of p85 upon p110 α catalytic activity (Burke et al. 2012). We carried out extensive molecular modeling of p.Ile112Asn onto the published crystal structure of p85-p110 α . Our findings strongly support the contention that p.Ile112Asn can weaken the ability of p110 α -ABD to effectively interact with the iSH2 domain of p85 α (Supp. Figure S4B-D and Supp. Structural Synopsis). Importantly, immunoprecipitation (IP) of p85 from patient cells consistently yielded less p110 α catalytic PI3K subunit compared to WT, providing functional validation using patient-derived material of the molecular impact/pathogenic nature of p.Ile112Asn (Figure 2H). This mechanistic explanation of PI3K hyperactivity has not previously been proposed as an underlying cause of congenital human disease (e.g. PROS/Cowden).

Analyzing the distribution of the *PIK3CA* mutations reported in both segmental overgrowth (PROS) and Cowden cohorts, it appears that *PIK3CA* mutations are distributed throughout the entire coding sequence of p110 α (Figure 1E). The C2 region is responsible for p110 α recruitment to cell surface membrane. C2-domain mutations have been shown to enhance recruitment of the mutant-p110 α to cell membranes, making lipid kinase activity independent of signals transmitted through the regulatory subunit, p85 (Gymnopoulos et al.

2007). Another mechanism of p110 α activation involving modifying protein-protein or protein-membrane interactions has been proposed for mutations located in the helicase domain, such as mutations at residue Glu545, which are observed as somatic mutations in different cancers (Gymnopoulos et al. 2007) as well as in segmental overgrowth (CLOVES, isolated macrodactyly, and hemimegalencephaly). A third activation mechanism has been described for mutations located in the kinase domain. These mutations cluster close to the hinge region of the activation loop and may induce a gain-of-function by changing the position and mobility of the activation loop.

Our findings compliment those of Burke and colleagues who have shown that certain cancer-associated mutations in the linker can enhance movement of the ABD region relative to the catalytic domain and in breaking the C2-iSH2 interface (Burke et al. 2012). Our functional data suggest a particular activation mechanism pertaining to the p.Ile112Asn mutation; altered stoichiometry within the p85-p110 complex and likely consequent weakening of the inhibitory effect of p85 upon p110. Interestingly, this mechanism may also apply for mutations p.Glu81Lys and p.Arg88Gln in p110 α -ABD (Supp. Figure S2A and Supp. Structural Synopsis). The latter was reported in two independent patients; as a mosaic mutation in one (LR11-068) as well as possibly a constitutive germline mutation in the other (LR06-220) (Mirzaa et al. 2012, Riviere et al. 2012). Remarkably, patient LR06-220 had severe macrocephaly (OFC +5 SD) but did not show body or brain asymmetry or somatic overgrowth frequently observed in MCAP patients with mosaic *PIK3CA* mutations. Her polymicrogyria was mild (bilateral perisylvian polymicrogyria grade 4) and intelligence assessed as normal; findings overlapping with the features observed in the patient presented here.

In summary, we describe a novel hyper-activating and constitutional *PI3KCA* mutation in an individual presenting with symmetric congenital macrocephaly and mild macrosomia at birth and lacking vascular and digital anomalies, thereby expanding upon the recent clinical spectrum proposed under the PROS umbrella. Using patient-derived cell lines, we demonstrate hyperactive PI3K and elevated PI3K-AKT-mTOR signaling associated with p.Ile112Asn. Molecular modeling of the 3-dimensional crystal structure of p85-p110 α suggests diminished interaction between p110 α and its negative regulatory subunit p85 could underlie the hyperactive PI3K activity. Using IP of p85 and subsequent Western blot analysis for p110 α from patient cells, we confirmed the prediction of the structural analysis: that pIle112Asn disrupts p85-p110 α interaction. This work illustrates the importance of integrating accurate phenotyping, genetic analysis, structural modeling and functional characterisation of patient cells using established endpoints to probe the nature of *PIK3CA* variants. Importantly, the patient reported here expands upon the known clinical presentation of hyper-activating *PIK3CA* mutations in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Patient images and *PIK3CA* mutational spectrum in PROS

Patient at 10 weeks of age. **B.** Patient at 8 months. **C.** Patient at 5 years: note high forehead, absence of other minor facial and digital anomalies, long halluces with bilateral sandal gap. **D.** Brain MRI at the age of 5 years. Note thick corpus callosum, large cerebellar vermis and a unilateral area suggestive for mild polymicrogyria (marked with asterisk). **E.** The mutation found in our patient is shown as a read box (Ile112Asn) on protein level (top) as well as on DNA level (below). All other mutations previously reported in “Cowden/Cowden-like” cohort (brown text) or as predominantly mosaic mutations by segmental overgrowth-PROS (no boxes). The blue text shows constitutional mutations in segmental overgrowth. Exons are drawn to scale but introns are shortened to 150 bp on either side of the exons. Protein domain annotation is based on <http://www.uniprot.org/uniprot/P42336>.

Figure 2. Functional analysis of patient-derived cells demonstrating hyperactive PI3K and elevated PI3K-AKT-mTOR pathway activation

A. Increasing amounts of whole cell extract (WCE) from wild-type (WT) and patient-derived lymphoblastoid cells (LCLs) were blotted for p110 α expression and the blots were re-probed for pan-p85 expression. No significant difference in expression of either protein was noted. **B.** Primary fibroblasts (FBs) were subjected to indirect immunofluorescence for PIP3. Elevated levels of PIP3 were observed in patient FBs. **C.** Acidic lipid fraction extracted from LCLs were dot blotted onto a nitrocellulose membrane and then probed with anti-PIP3. Signal intensity quantified using Image J (a.u.; arbitrary units) and represented as a bar chart. Error bars represent mean $\pm$ s.d (n=3). MCAP LCLs from *PIK3CA*-mutated individual (p.Glu453del) were included for comparison (*p<0.05 Student's t test). We have previously shown elevated levels of PIP3 in these cells (LR05-204) (Riviere et al 2012). *PIK3CA*-mutated LCLs (p110 α : p.Ile112Asn) exhibited elevated PIP3 signal compared to WT LCLs (*p<0.05 Student's t test), although this was somewhat lower than the MCAP LCLs. **D.** Primary FBs were assessed for levels of phospho-S6 (S6-pS240/244), a terminal event of the PI3K-AKT-mTOR signaling pathway. FBs were serum starved (48hrs. NS; no serum) and WCE titrated and probed for S6-pS240/244 (upper left hand panel). Elevated basal levels of S6-pS240/244 were found in the patient FBs relative to WT. Acute treatment with EGF (10nM 10mins) increased S6-pS240/244 signal in WT FBs, although induction was even higher in the patient FBs. These results are consistent with elevated PI3K-AKT-mTOR signaling in the patient cells. The panels on the right hand side represent β -tubulin re-probes to confirm equal loading. **E.** Signal intensity for S6-pS240/244 quantified using Image J (a.u.; arbitrary units) and represented as a bar chart with error bars represent mean $\pm$ s.d (n=3) (*p<0.05 Student's t test). **F.** Exponentially growing WT and patient LCLs were treated (2hrs) with increasing concs of PI-103 (0, 50, 100, 200nM) prior to extraction. Note the higher S6-pS240/244 signal at 0 nM in the patient extract compared to WT indicative of elevated endogenous PI3K-AKT-mTOR signaling. WCE was titrated and probed for S6-pS240/244 expression. At higher PI-103 concs the signal was diminished in WT LCLs compared to patient LCLs. This relative insensitivity of the patient LCLs to PI-103

treatment reflects the elevated PI3K-AKT-mTOR signaling characteristic of these cells. **G.** Primary FBs were serum starved (48hrs) and WCE probed for AKT-pS473 in the absence of (–) and following acute treatment with EGF (10nM, 10mins). Elevated AKT-pS473 was evident in patient FBs under both conditions, compared to WT cells. **H.** p85 was immunoprecipitated (IP) from LCLs (upper panels) and primary FBs (lower panels) and blots probed for p110 α levels. IgH: immunoglobulin heavy chains. We observed less p110 α in immunoprecipitates from patient-derived LCLs and primary FBs compared to WT, consistent with a weakened interaction between p85 and p110 α .