A Novel *MYCN* Variant Associated with Intellectual Disability Regulates Neuronal Development

Xiuya Yu¹ · Liyuan Hu¹ · Xu Liu¹ · Guodong Zhan¹ · Mei Mei¹ · Huijun Wang² · Xiaohua Zhang³ · Zilong Qiu⁴ · Wenhao Zhou^{1,2} · Lin Yang^{2,5}

Received: 3 November 2017 / Accepted: 17 April 2018 / Published online: 21 May 2018
© Shanghai Institutes for Biological Sciences, CAS and Springer Nature Singapore Pte Ltd. 2018

Dear Editor,

The V-MYC avian myelocytomatosis viral-related oncogene, a neuroblastoma-derived gene (*MYCN*, MIM: 164840) located on chromosome 2p24, was previously found to be associated with Feingold syndrome 1 (FGLDS1, MIM: 164280) [1]. FGLDS1 is an autosomal dominant disorder characterized by variable combinations of microcephaly, limb malformations, esophageal and duodenal atresias, and learning disabilities. Cardiac and renal malformations, vertebral anomalies, and deafness have also been described in a minority of patients [2]. Despite the involvement of intellectual disability in FGLDS1, the molecular mechanisms of the *MYCN* gene in regulating brain development remain largely unclear.

Xiuya Yu and Liyuan Hu have contributed equally to this work.

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s12264-018-0236-4>) contains supplementary material, which is available to authorized users.

✉ Lin Yang
yanglin_fudan@163.com

- ¹ Department of Neonatology, Children's Hospital of Fudan University, Shanghai 201102, China
- ² Key Laboratory of Birth Defects, Children's Hospital of Fudan University, Shanghai 201102, China
- ³ Department of Neurosurgery, Renji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai 200127, China
- ⁴ Institute of Neuroscience, Shanghai Institute of Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China
- ⁵ Department of Genetics, Children's Hospital of Fudan University, Shanghai 201102, China

Some truncated mutations in the N terminus of the *MYCN* have been identified in FGLDS1 [1, 3].

In this study, a Chinese girl affected by frontal bossing, hypertelorism, a high-arched palate, speech delay, and intellectual disabilities was recruited. We found a missense mutation in the *MYCN* gene, which explained the less severe symptoms of FGLDS1 but more symptoms of developmental disorder including intellectual disabilities. Thus, we propose to classify the patient as having non-canonical FGLDS1, and hypothesize that the missense mutation of *MYCN* contributes to the symptoms through alterations in brain development.

Whole-exome sequencing to a median of 50 × coverage identified 98671 genetic variants in the proband. After filtering, 448 rare variants in 433 genes were identified. A negative family history suggested *de novo* or autosomal recessive inheritance, and only variants located in morbid genes according to the Online Mendelian Inheritance in Man (OMIM) database and fitting in the inheritance model were identified as potential pathogenic mutations. Finally, a total of 3 variants in 3 genes were selected (Table 1).

A *de novo* heterozygous variant in the *MYCN* gene (NM_005378, exon2, c.G550T, p.A184S) was identified in the proband. The *MYCN* mutation was further validated by Sanger sequencing. This *MYCN* A184S variant was not found in the parents, so it was defined as a *de novo* variant (Fig. 1A). Other two disorder-related genes were identified in the proband: *MEGF8* [MIM: 604267] and *PDE4D* [MIM: 600129] (Table 1). The protein encoded by the *MEGF8* gene [multiple EGF (epidermal growth factor)-like-domains 8] is a single-pass type I membrane protein that contains several EGF-like domains. Defects in this gene cause Carpenter syndrome, which is an autosomal recessive multiple congenital malformation disorder characterized by multisuture craniosynostosis and polysyndactyly, umbilical

Table 1 Summary of morbid OMIM genes with *de novo* variants in the proband.

Gene	Chr	Location	NM	Mutation call	Hom/ Het	Disease
<i>MYCN</i>	2	16082736	NM_005378	c.550G > T:p.Ala184Ser	Het	Feingold syndrome [MIM: 164280]
<i>MEGF8</i>	19	42857691	NM_001271938	c.3625C > T:p.Arg1209Trp	Het	Carpenter syndrome 2 [MIM: 614976]
<i>PDE4D</i>	5	58286737	NM_001165899	c.1006-8delCinsTC	Het	Acrodysostosis 2, with or without hormone resistance [MIM: 614613]

hernia, cryptorchidism, and congenital heart disease [4]. Genetic variations in *PDE4D* might be associated with susceptibility to stroke [5]. *PDE4D* is known to be associated with acrodysostosis 2 (ACRDYS2) [MIM: 614613], a pleiotropic disorder characterized by skeletal, endocrine, and neurological abnormalities. The skeletal features include brachycephaly, midface hypoplasia with a small upturned nose, brachydactyly, and lumbar spinal stenosis. The endocrine abnormalities include hypothyroidism and hypogonadism in males and irregular menses in females [6, 7]. The main clinical manifestations of the *MEGF8* and *PDE4D* genes were not consistent with those of our proband, so we focused on the variant in the *MYCN* gene as the most likely disease-causing event.

To investigate the role of the MYCN protein during neuronal development, we first examined the expression pattern of MYCN during mouse brain development. We found that the MYCN protein was consistently expressed during postnatal days 0–35 in the cortical region (Fig. 1B). *MYCN* is a critical tumor-related gene and may be associated with numerous tumor suppressors, including p53 and PTEN [8]. Thus, we cloned the human wild-type *MYCN* gene and added the A184S mutation to investigate the function of MYCN in neuronal development.

First, we determined whether MYCN regulated p53 and PTEN expression by overexpressing MYCN in human embryonic kidney (HEK293) cells and mouse primary cortical neurons. Interestingly, we found that MYCN significantly repressed the expression of p53 and PTEN in the HEK293 cells at the mRNA level (Fig. 1C, D). Furthermore, we verified at the protein level that overexpression of MYCN suppressed PTEN as well (Fig. S1A, B). Importantly, the A184S mutation of *MYCN* significantly inhibited PTEN expression in mouse cortical neurons, while the wild-type (WT) *MYCN* did not, suggesting that A184S is a gain-of-function mutation for *MYCN* (Fig. 1E).

Next, we investigated the roles of WT and A184S-mutated *MYCN* in neuronal development. Surprisingly, we found that *MYCN* overexpression and the A184S *MYCN* mutation significantly enhanced the length of axons (Fig. 1F, G), further suggesting that MYCN plays a critical role in regulating the development of neuronal morphology and that A184S is likely to be a gain-of-function mutation.

Then we designed short-hairpin RNA against the mouse *Mycn* gene to confirm the role of Mycn in neuronal development (Fig. 1H). The shRNA against *Mycn* was transfected along with the vector into mouse primary cortical neurons and the neuronal morphology was observed after 2–3 days. Consistently, knockdown of *Mycn* strongly inhibited axonal and neurite growth (Fig. 1I, J), indicating that Mycn plays a bi-directional role in regulating neuronal morphology.

To further define the functional differences between the WT and the A184S MYCN mutation, we collected HEK293 cell samples transfected with *MYCN* and *MYCN* A184S vectors and sent them for RNA sequencing. Significant differences in the expression patterns between the WT and the A184S group were found (Fig. 2A). Interestingly, Gene Set Enrichment Analysis (GSEA) showed that the A184S mutation group had greater activation in several pathways, such as the axon guidance, PI3K-AKT activation, and Wnt signaling pathways (Fig. 2B). We then analyzed the differences in microRNA expression between the two groups (Table S1) and obtained predicted target genes using the Miranda and RNAhybrid analysis methods. The target genes were further identified using the Fisher analysis method to classify pathways using Gene Ontology analysis. Notably, the axon guidance, Wnt signaling, and PI3K-AKT signaling pathways were among the top 15 (Fig. 2C, D), suggesting that the A184S mutation has an enhancing effect on axonal growth.

In this report, we present a female patient with frontal bossing, hypertelorism, a high-arched palate, speech delay, and mental retardation. The patient carries a transition of c.G550T, which causes a heterozygous mutation of p.A184S in exon 2 of the *MYCN* gene. Furthermore, this is a *de novo* mutation validated by Sanger sequencing. Until now, all of the identified mutations mapped within the repressor domain have been nonsense mutations (E73X, W77X, G151X, and S221X) or frame-shift mutations (E47fs, G101fs, and P228fs), which would remove most regions of the wild-type protein, as in haploinsufficiency, which induces FGLD1 [1, 3, 9, 10]. However, we identified a missense mutation of p.A184S in this region. This mutation is a milder functional defect than haploinsufficiency, so it may contribute to a less severe and atypical phenotype of FGLD1.

Fig. 1 Expression pattern of *MYCN* in mouse brain and its role in neural development. **A** Known nonsense mutations of the *MYCN* gene that cause FGLDS1 (green and black) and the newly-found mutation (red). Sanger sequencing results confirmed that the mutation was *de novo*. **B** Western blots of *MYCN* protein levels in the cortical region of P0-P35 mice. **C**, **D** Quantitation of *PTEN* (**C**) and *P53* (**D**) levels in HEK293 cells transfected with control (CTRL), *MYCN*, and *MYCN*-A184S plasmids. RNA samples were collected 48 h after plasmid transfection, and levels were assessed by qPCR. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. **E** Quantitation of *PTEN* levels in mouse primary cortical neurons transfected with CTRL, *MYCN*, and *MYCN*-A184S plasmids. RNA samples were collected 5 days after plasmid transfection. $**P < 0.01$. **F**, **G** Representative images (**F**) and statistics for axonal length (**G**) of mouse primary cortical neurons

transfected with CTRL, *MYCN*-WT, and *MYCN*-A184S. Neurons were fixed and immunostained with GFP antibody at 3 DIV for measurement of axon length. More than 70 examples in each group of neurons were randomly selected for counting. $***P < 0.001$. **H** Quantitation of *MYCN* mRNA levels in mouse cortical neurons transfected with sh-DsRed-GFP (CTRL) and *MYCN*-shRNA (sh-*MYCN*); mRNA levels were assessed by qPCR. RNA samples were collected 5 days after plasmid transfection. $**P < 0.01$. **I** Representative images of mouse primary cortical neurons transfected with sh-DsRed-GFP (CTRL) or sh-*MYCN*. Neurons were fixed and immunostained with GFP antibody at 3 DIV. **J** Measurements of axonal length in the sh-DsRed-GFP (CTRL) and sh-*MYCN* groups. More than 50 examples from each group of neurons were randomly selected for counting. $***P < 0.001$.

Fig. 2 mRNAs and microRNAs were differentially expressed in the *MYCN*-A184S group compared to HEK293 *MYCN*-WT cells. **A** Heatmap of differentially-expressed mRNAs between the *MYCN*-A184S and *MYCN*-WT groups; each group contained four samples (false discovery rate < 0.05, fold change > 2). **B** Gene set enrichment analysis of the axon guidance, PI3K-AKT activation, and Wnt

signaling pathways in the *MYCN*-A184S and *MYCN*-WT groups at the mRNA level. **C** Pathway analysis of target genes predicted by differentially-expressed miRNAs in the *MYCN*-A184S group compared to the *MYCN*-WT group. **D** Gene ontology (GO) analysis of target genes predicted by differentially-expressed miRNAs in the *MYCN*-A184S group compared to the *MYCN*-WT group.

In total, 488 rare variants in 433 genes were identified in the proband after the filtering step. We focused on the variants that were located in the morbid OMIM genes and

fitted into the inheritance model in this three-person family without any known family history. Although genes without clear associations with the specific phenotype may

contribute to the clinical manifestations of the proband, such variants need more comprehensive evidence to confirm their relevance.

MYCN is a downstream target of Shh signaling, which promotes the rapid cell division of granule neuron progenitors (GNPs) in mice [11]. Overexpression of MYCN promotes the proliferation of GNPs independent of Shh signaling, and conversely, its conditional deletion during embryonic cerebellar development results in severe GNP deficiency, perturbation of foliation, and reduced cerebellar mass [11]. Valli *et al.* have shown that a functional axis between MYCN and cyclin-dependent kinase-like 5 governs both the neuron proliferation rate and differentiation, potentially contributing to the neurological symptoms in patients with MYCN mutations [12].

Maintaining the normal morphology of neurons is crucial for basic brain functions, since axons play important roles in structural formations and signal transmission. Dysfunctional axons may cause many neurological disorders, such as autism spectrum disorders [13]. In this study, we focused on the role of dysfunctional axons in the intellectual disability found in FGLDS1, and found that overexpression of MYCN strongly promoted axonal growth, whereas its knockdown repressed the axonal growth of mouse cortical neurons. Interestingly, the A184S mutation in MYCN identified in this patient with non-canonical FGLDS1 syndrome had a stronger effect on axonal growth.

Furthermore, we found that the A184S mutation significantly inhibited the expression of p53 and PTEN, according to the RNA sequencing results. The GSEA pathway and GO analysis indicated that the A184S mutation has a greater effect on the axonal guidance, PI3K-AKT activation, and Wnt signaling pathways. PTEN is a known important component in neurite growth *via* regulating the PI3K-AKT-mTORC1 signaling pathway, and its downregulation promotes neurite growth [14], which was also shown in our results. The Wnt receptor in Wnt signaling is also associated with axonal guidance [15]. These findings suggest that A184S is a gain-of-function mutation.

Although further research is still needed, our study suggests a possible role of the A184S mutation in intellectual disability. The results shed new light on the mechanistic insights of MYCN-related brain developmental disorders and provide a potential candidate for mutation screening in related brain disorders.

Acknowledgements We express our deep gratitude to the patient and her family for their willingness to participate and sustained cooperation in the study. This work was supported by grants from the National Natural Science Foundation of China (81701494), the Shanghai Municipal Commission of Health and Family Planning (2013ZYJB0015), and the Science and Technology Commission of Shanghai Municipality (14411950402).

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no competing financial interests.

References

1. van Bokhoven H, Celli J, van Reeuwijk J, Rinne T, Glaudemans B, van Beusekom E, *et al.* MYCN haploinsufficiency is associated with reduced brain size and intestinal atresias in Feingold syndrome. *Nat Genet* 2005, 37: 465–467.
2. Teszas A, Meijer R, Scheffer H, Gyuris P, Kosztolanyi G, van Bokhoven H, *et al.* Expanding the clinical spectrum of MYCN-related Feingold syndrome. *Am J Med Genet A* 2006, 140: 2254–2256.
3. Marcelis CL, Hol FA, Graham GE, Rieu PN, Kellermayer R, Meijer RP, *et al.* Genotype-phenotype correlations in MYCN-related Feingold syndrome. *Hum Mutat* 2008, 29: 1125–1132.
4. Twigg SR, Lloyd D, Jenkins D, Elcioglu NE, Cooper CD, Al-Sannaa N, *et al.* Mutations in multidomain protein MEGF8 identify a Carpenter syndrome subtype associated with defective lateralization. *Am J Hum Genet* 2012, 91: 897–905.
5. Nilsson-Ardnor S, Wiklund PG, Lindgren P, Nilsson AK, Janunger T, Escher SA, *et al.* Linkage of ischemic stroke to the PDE4D region on 5q in a Swedish population. *Stroke* 2005, 36: 1666–1671.
6. Lee H, Graham JM, Jr., Rimoin DL, Lachman RS, Krejci P, Tompson SW, *et al.* Exome sequencing identifies PDE4D mutations in acrodysostosis. *Am J Hum Genet* 2012, 90: 746–751.
7. Michot C, Le Goff C, Goldenberg A, Abhyankar A, Klein C, Kinning E, *et al.* Exome sequencing identifies PDE4D mutations as another cause of acrodysostosis. *Am J Hum Genet* 2012, 90: 740–745.
8. Zheng H, Ying H, Yan H, Kimmelman AC, Hiller DJ, Chen AJ, *et al.* p53 and Pten control neural and glioma stem/progenitor cell renewal and differentiation. *Nature* 2008, 455: 1129–1133.
9. Blaumeiser B, Oehl-Jaschkowitz B, Borozdin W, Kohlhasse J. Feingold syndrome associated with two novel MYCN mutations in sporadic and familial cases including monozygotic twins. *Am J Med Genet A* 2008, 146A: 2304–2307.
10. Cognet M, Nougayrede A, Malan V, Callier P, Cretolle C, Faivre L, *et al.* Dissection of the MYCN locus in Feingold syndrome and isolated oesophageal atresia. *Eur J Hum Genet* 2011, 19: 602–606.
11. Oliver TG, Gräsfeder LL, Carroll AL, Kaiser C, Gillingham CL, Lin SM, *et al.* Transcriptional profiling of the Sonic hedgehog response: a critical role for N-myc in proliferation of neuronal precursors. *Proc Natl Acad Sci U S A* 2003, 100: 7331–7336.
12. Valli E, Trazzi S, Fuchs C, Erriquez D, Bartesaghi R, Perini G, *et al.* CDKL5, a novel MYCN-repressed gene, blocks cell cycle and promotes differentiation of neuronal cells. *Biochim Biophys Acta* 2012, 1819: 1173–1185.
13. Lazar M, Miles LM, Babb JS, Donaldson JB. Axonal deficits in young adults with High Functioning Autism and their impact on processing speed. *Neuroimage Clin* 2014, 4: 417–425.
14. Hsia HE, Kumar R, Luca R, Takeda M, Courchet J, Nakashima J, *et al.* Ubiquitin E3 ligase Nedd4-1 acts as a downstream target of PI3K/PTEN-mTORC1 signaling to promote neurite growth. *Proc Natl Acad Sci U S A* 2014, 111: 13205–13210.
15. Clark CE, Liu Y, Cooper HM. The Yin and Yang of Wnt/Ryk axon guidance in development and regeneration. *Sci China Life Sci* 2014, 57: 366–371.