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Case report

A recurrent, non-penetrant sequence variant, p.Arg266Cys in Growth/Differentiation Factor 3 (*GDF3*) in a female with unilateral anophthalmia and skeletal anomalies

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ABSTRACT

Purpose: The genetic causes of anophthalmia, microphthalmia and coloboma remain poorly understood. Missense mutations in Growth/Differentiation Factor 3 (*GDF3*) gene have previously been reported in patients with microphthalmia, iridial and retinal colobomas, Klippel-Feil anomaly with vertebral fusion, scoliosis, rudimentary 12th ribs and an anomalous right temporal bone. We used whole exome sequencing with a trio approach to study a female with unilateral anophthalmia, kyphoscoliosis and additional skeletal anomalies.

Observations: Exome sequencing revealed that the proposita was heterozygous for c.796C > T, predicting p.Arg266Cys, in *GDF3*. Sanger sequencing confirmed the mutation and showed that the unaffected mother was heterozygous for the same missense substitution.

Conclusions and importance: Although transfection studies with the p.Arg266Cys mutation have shown that this amino acid substitution is likely to impair function, non-penetrance for the ocular defects was apparent in this family and has been observed in other families with sequence variants in *GDF3*. We conclude p.Arg266Cys and other *GDF3* mutations can be non-penetrant, making pathogenicity more difficult to establish when sequence variants in this gene are present in patients with structural eye defects.

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1. Introduction

Anophthalmia (absent eyes) and microphthalmia (small eyes) are important birth defects because of the medical significance of severely reduced vision. Coloboma, or a failure of the optic fissure to close, is a common accompaniment to microphthalmia. Mutations in *SOX2* and genes coding for other transcription factors can cause microphthalmia, anophthalmia and coloboma (MAC), but there is high genetic heterogeneity[1,2] and more than 50% of affected individuals remain without a molecular genetic diagnosis for their birth defect after sequencing of the currently known causative genes.[3–6] Mutations in the Growth/Differentiation Factor 3 (*GDF3*) gene were first described as a cause of microphthalmia and coloboma after five, heterozygous sequence variants that cause single amino acid substitutions were identified in seven individuals from a cohort of 472 patients with MAC.[7] This frequency of *GDF3* sequence variants (7/472 or 1.5%) was significantly increased in MAC patients compared to controls ($p = 0.007$) for that study.[7] Affected individuals exhibited a spectrum of ocular and skeletal findings comprising unilateral or bilateral microphthalmia and coloboma with involvement of the iris and retina, vertebral defects with Klippel-Feil syndrome and thoracic and lumbar scoliosis, rudimentary 12th ribs and an anomalous right temporal bone. There was high intrafamilial and interfamilial variability.[7]

One recurrent mutation in *GDF3*, c.796C > T, predicting p.Arg266Cys, segregated with four affected members of a three-generation pedigree, two affected members of a two-generation pedigree, and was present in an individual with no affected relatives. p.Arg266Cys affects a well-conserved arginine residue

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located in the mature TGF-β domain (Table 1) and the mutation introduces a cysteine residue that is predicted to enable the formation of an aberrant disulfide bridge between residues C266 and C329 of the GDF3 protein to prevent stabilization of the TGF-β fold.[7] Transfection of COS-7 cell lysates and a *SOX9*-based reporter assay revealed an appreciable reduction in mature, secreted GDF3 ligand on Southern blot, with diminished expression of the downstream *SOX9* gene for mutant p.Arg266Cys *GDF3* compared to wildtype *GDF3*.[7]

As part of a study into the genetic etiology of anophthalmia and microphthalmia, we sequenced a female with unilateral microphthalmia and skeletal defects comprising pectus excavatum, scoliosis, enhanced kyphosis and finger contractures. We identified the recurrent *GDF3* sequence variant, c.796C > T, predicting p.Arg266Cys, that was inherited from the proposita's healthy mother. We report this patient and review the evidence that this and other reported missense variants in *GDF3* are pathogenic in MAC.

2. Case report

The pregnancy with the proposita was normal and she was delivered using forceps for failure to progress. She was noted to have unilateral right anophthalmia/severe microphthalmia, with a small residual eye remnant described as the size of 'a raisin.' Congenital hip dislocation was treated with a cast for 2 weeks and resolved at 3–4 months of age. She started talking at one year of life and walked at 18 months of age. She had normal intelligence and was an excellent student who learned to read at 4–5 years of age. She is currently 20 years of age and is active and able to play sports and continue her studies. Her depth perception is reportedly normal.

She was diagnosed with pectus excavatum, scoliosis and bilateral finger contractures at 11 years of age. She developed chest asymmetry with a smaller right breast, but has not required surgery for her pectus deformity. Her other medial history included severe dry skin, asthma treated with inhalants, and headaches. She required an incision to aid emergence of an upper left canine. She had menarche at 11 years of age. At 20 years of age, her height was 170 cm (50–75th centile) and weight was 53 kg (10–25th centile). She had clubbing of the fingernails that was most marked for her second fingers and less evident for her fifth digits. A karyotype and microarray have not been performed.

Both of the proband's (IV-1) parents (III-2 and III-3) were healthy and there was no known consanguinity (Fig. S1). Ethnicity was Caucasian. A maternal great-uncle (II-5) was blind in one eye, but remembered having a small amount of vision in that eye as a young child and has not had known structural eye defects; 'degeneration' was mentioned by the physician but he has not received a diagnosis. He had a maternal aunt (I-4) and a paternal great-aunt (not shown) with a similar condition by report. A maternal aunt had normal vision, but her son (IV-2) reportedly had anophthalmia/microphthalmia, although further details are unknown. A maternal cousin (III-1) had amblyopia by report. DNA testing on these relatives has not been performed.

2.1. Exome sequencing

After obtaining written, informed consent, venous blood samples were obtained and DNA was extracted from the proposita and both parents. Exome sequencing was performed as previously described.[8,9] We utilized wAnnovar (http://wannovar.usc.edu/) with default parameters to generate an annotated variant call file (.vcf file).[10,11] We analyzed the .vcf file using Station X GenePool software (http://www.stationxinc.com). We used SnpEff (http://snpeff.sourceforge.net/SnpEff_manual.html) to filter for highly deleterious and moderately deleterious variants that had a European allele frequency of less than 1% according to 1000 Genomes Database (http://www.1000genomes.org/). The variant list was then filtered for eye disease genes based on disease ontology annotations. The potential deleteriousness of novel sequence variants was assessed using SIFT (http://sift.jcvi.org/),[12] Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/)[13] and Mutation Taster (http://mutationtaster.org/MutationTaster/).[14]

A mean target coverage of 96X was obtained, with 94.6% of target sequence covered at 10X (Table S1). We first examined the data for sequence variants in genes previously published as causative for anophthalmia and microphthalmia.[1,2] In *GDF3*, we observed a recurrent sequence variant that has previously been published as an autosomal dominant mutation for patients with microphthalmia and coloboma, c.796C > T predicting p.Arg266Cys (NM_020634.1).[7] We used Sanger sequencing to verify the

Table 1
Characteristics of p.Arg266Cys and other sequence variants in Growth/Differentiation Factor 3 (*GDF3*).

Nucleotide alteration	Amino acid alteration	Protein domain	SIFT[a]	PolyPhen-2[b]	Mutation Taster[c]	1000 Genomes[d]	ExAC Browser[e]	dbSNP[f]
c.584G > A	p.Arg195Gln	Pre-pro domain	0.54	0.001; B[g]	PM[h]; p = 0.999	1/1000; 0.001; het.[i]	65/121,408; 0.0005354; HZ[j] = 0	rs146973734; 0.001 ± 0.026
c.796C > T	p.Arg266Cys	Transforming growth factor-β, C-terminal	0.01	0.24; PM	DC[k]; p = 0.999	2/1000; 0.002; het.	246/121,398; 0.002026; HZ = 0	rs140926412; 0.003 ± 0042
c.820C > T	p.Arg274Trp	Transforming growth factor-β, C-terminal	0	0.99	PM; p = 0.995	2/1000; 0.002; het.	30/121,400; 0.0002471; HZ = 1	rs387906946; 0.000 ± 0.016
c.914T > C	p.Leu305Pro	Transforming growth factor-β, C-terminal	0	1.0	DC[h]; p = 0.999	8/1000; 0.008; het.	82/121,408; 0.0006754; HZ = 1	rs387906945; 0.001 ± 0.026
c.974C > T	p.Pro325Leu	Transforming growth factor-β, C-terminal	0.0002	1.0	DC[h]; p = 0.999	1/1000; 0.001; het.	14/121,404; 0.0001153; HZ = 0	rs566697767; 0.000 ± 0.011

[a] SIFT = Sorting Intolerant from Tolerant, http://sift.jcvi.org/.
[b] Polyphen-2 = http://genetics.bwh.harvard.edu/pph2/.
[c] Mutation Taster = http://www.mutationtaster.org.
[d] 1000 Genomes = http://www.1000genomes.org.
[e] ExAC Browser = http://exac.broadinstitute.org.
[f] dbSNP = http://www.ncbi.nlm.nih.gov/SNP/.
[g] B = benign.
[h] PM = polymorphism.
[i] het. = heterozygous.
[j] HZ = homozygous.
[k] DC = disease causing.

Table 2
Conservation of p.Arg266Cys in Growth/Differentiation Factor 3 (*GDF3*).

Species	Gene	Alignment for p.(Arg266Cys)
H.sapiens Wildtype	ENST00000329913	V P K L S C K N L C H **R** H Q L F I N F R D L G
H.sapiens Mutated	ENST00000329913	V P K L S C K N L C H **C** H Q L F I N F R D L G
P.troglodytes	ENSPTRG00000004615	V P K V S C K N L C H **R** H Q L F I N F R D L G
M.mulatta	ENSMUSG00000030117	V P K V S C K N L C H **R** H Q L F I N F Q D L G
F.catus	ENSFCAG00000009940	A P K A P C R N L C H **R** H Q L F I N F R D L G
M.musculus	ENSMUSG00000030117	V P K G F C R N F C H **R** H Q L F I N F Q D L G
G.gallus	ENSGALG00000003161	V P - V T P S N L C K **P** R R L Y I S F S D V G
T.rubripes	ENSTRUG00000014765	L P - V T P S N V C K **A** R R L Y I D F K D V G
D.rerio	ENSDARG00000037995	V C K **P** R R L Y I D F K D V G
D.melanogaster	No homologue	
C.elegans	T25F10.2	N L C R **R** T D F Y V D F D D L N
X.tropicalis	ENSXETG00000002076	S P P T P - S N I C K **K** R R L Y I D F K D V G

sequence variant in the proposita and to test her parents, revealing that her unaffected mother carries the same variant (Fig. S2) and that her father does not have this variant. *GDF3* encodes a single transcript that yields a protein of 364 amino acids; Arg266 is located in the Transforming Growth Factor-β, C-terminal domain. Software predictions regarding p.Arg266Cys were mixed, with SIFT and MutationTaster predicting that the substitution was deleterious, whereas PolyPhen-2 predicted that the amino acid change was a benign polymorphism (Table 1). ExAC browser and dbSNP listed p.Arg266Cys as having an estimated frequency of 0.002–0.003 in normal controls, whereas the frequency in 1000 Genomes database was estimated at 0.0399361 (Table 1). Conservation of the Arg266 residue was noted in several species, including P.troglodytes, *M.mulatta*, *F.catus*, *M.musculus* and *C.elegans*, but the residue was not preserved in *G.gallus*, *T.rubripes*, *D.rerio* or *X.tropicalis* (Table 2). The mutation was not predicted to have an effect on splicing (MutationTaster).

In view of the inheritance of p.Arg266Cys from the proposita's unaffected mother, we hypothesized that genetic burden for deleterious sequence variants in additional genes for MAC could explain the difference in penetrance.[15] We used the same Station-X GenePool filtering parameters to compare the number of predicted deleterious sequence variants in the listed genes associated with MAC[1,2] in the proposita and both parents. We did not observe an increased burden of deleterious sequence variants in these genes in the proposita compared to her mother (Table 3), although this analysis was limited and does not include sequence variants in novel genes or copy number variants that were undetected with our exome analysis. Further testing of additional family members was not possible.

3. Discussion

We identified a sequence variant in *GDF3*, c.796C > T predicting p.Arg266Cys, in a female with unilateral anophthalmia and skeletal anomalies comprising pectus excavatum, scoliosis, kyphosis and bilateral finger contractures. This sequence variant has previously been published as a causative mutation for both structural eye defects and skeletal anomalies; however, non-penetrance has been described for this variant and was present in this family, assuming this variant is pathogenic.[7] GDF3 is a member of the TGF-β superfamily and is classified as a BMP/GDF ligand, although it is missing the fourth of seven canonical cysteine residues that define the cysteine knot for members of the TGF-β superfamily.[16] GDF3 has been shown to inhibit bone morphogenetic protein (BMP) signaling in human and mouse embryonic stem (ES) cells and frog embryos.[16,17] The inhibition of BMP in these studies was executed by processed and unprocessed forms of GDF3, and it has been hypothesized that the "missing cysteine" residue in GDF3 was a critical component for the inhibitory activity of BMP signaling.[16,18] However, the "missing" fourth cysteine motif is required for GDF3 function only if the cleavage of the prepro domain is also mutated to impair maturation of the GDF3 protein.[16] At high doses, GDF3 has been shown to act as a Nodal-like ligand, stimulating activity of Smad 2/3.[16]

We reviewed the frequency and predicted deleteriousness of published sequence variants in *GDF3* that are predominantly found in one publication (Table 4). In studies of MAC in which *GDF3* was sequenced, five missense sequence variants found in a cohort of 472 patients accounted for 1.7% of individuals screened[19] and *GDF3* variants thus remain rare as putative causes of MAC.[20] Three of the above amino acid substitutions in *GDF3* were predicted to be benign or a polymorphism by at least one software prediction program (Table 1); all of the published missense variants have been detected in control databases, with two (c.820C > T, predicting p.Arg274Trp, and c.914T > C, predicting p.Leu305Pro) being present in a homozygous state in these databases (Table 1). No nonsense or frameshift mutations in *GDF3* have been published in association with eye defects.[20] Incomplete penetrance was also present in our family and in a family with a different mutation in the *GDF3* gene, c.914T > C, predicting p.Leu305Pro, in which two individuals carried this missense substitution, but only one individual manifested

Table 3
Mutations in known causative genes for eye defects in the proposita and unaffected parents.[a]

Gene	Nucleotide	Protein	Zygosity	1000 Genomes[b]	Predicted impact
Proposita					
GCNT2; NM_145649	c.305C > G	p.Thr102Ser	Het[c]	0.658946	Moderate
FANCD2; NM_001018115	c.1214A > G	p.Asn405Ser	Het.	–	moderate
GLI2; NM_005270	c.3943C > T	p.Pro1315Ser	Het.	0.998403	moderate
GDF3; NM_020634.1	c.796C > T	p.Arg266Cys	Het.	0.0399361	moderate
Mother					
CREBBP; NM_001079846	c.1537C > A	p.Leu513Ile	Het.	0.638978	moderate
PAX2; NM_000278	c.974C > T	p.Thr325Ile	Het.	–	moderate
TCOF1; NM_000356	c.1552G > A	p.Val518Ile	Het.	0.239617	moderate
MKS1; NM_001165927	c.1352A > G	p.Tyr451Cys	Het.	–	moderate
FANCD2; NM_001018115	c.1214A > G	p.Asn405Ser	Het.	–	moderate
GLI2; NM_005270	c.3943C > T	p.Pro1315Ser	Het.	0.998403	moderate
GDF3; NM_020634.1	c.796C > T	p.Arg266Cys	Het.	0.0399361	moderate
Father					
FANCD2; NM_001018115	c.1214A > G	p.Asn405Ser	Het.	–	moderate
ISPD; NM_001101417	c.407C > T	p.Ala136Val	Het.	0.938498	moderate
SALL1; NM_198097	c.477*>+CGA	p.Ser159X	Het.	–	moderate
GCNT2; NM_145649	c.305C > G	p.Thr102Ser	Het.	0.658946	moderate
ODZ3; NM_001080477	c.6682C > T	p.Arg2228Cys	Het.	0.0399361	moderate

[a] Variants were filtered to retain those with a Minor Allele Frequency of 1%.
[b] 1000 Genomes http://www.1000genomes.org.
[c] Het. = heterozygous.

Table 4
Previously reported sequence variants in Growth/Differentiation Factor 3 (*GDF3*).

Phenotype	Mutation	Penetrance/Segregation	Reference
Ocular			
Microphthalmia, coloboma	c.974C > T, p.Pro325Leu	Unaffected father	Prokudin et al., 2014
Bilateral coloboma, microphthalmia, nystagmus	c.796C > T, p.Arg266Cys[1]	Family #3; Segregation in 2 generations	Ye et al., 2010
Bilateral coloboma, mild microphthalmia,	c.796C > T, p.Arg266Cys[1]	Family #3; Segregation in 2 generations	Ye et al., 2010
Unilateral microphthalmia	c.914T > C, p.Leu305Pro	Family #2; Incomplete	Ye et al., 2010
Bilateral iris coloboma	c.914T > C, p.Leu305Pro	Proband 7; Unknown	Ye et al., 2010
Unilateral microphthalmia	c.584G > A, p.Arg195Gln	Proband 5; Autosomal dominant	Ye et al., 2010
Bilateral microphthalmia and coloboma	c.820C > T, p.Arg274Trp	Proband 6; Unknown	Ye et al., 2010
Skeletal			
Scoliosis	c.796C > T, p.Arg266Cys	Family #1; Segregation in 3 generations	Ye et al., 2010
Klippel-Feil and vertebral fusion	c.796C > T, p.Arg266Cys	Family #1; Segregation in 3 generations	Ye et al., 2010
Klippel-Feil and vertebral fusion	c.796C > T, p.Arg266Cys	Family #1; Segregation in 3 generations	Ye et al., 2010
Combined			
Unilateral iris and retinal coloboma, rudimentary 12th ribs, mild scoliosis	c.796C > T, p.Arg266Cys	Family #1; Segregation in 3 generations	Ye et al., 2010
Bilateral coloboma, microphthalmia, Anomalous right temporal bone	c.796C > T, p.Arg266Cys[1]	Proband 4 Unknown	Ye et al., 2010

1. In the first family, the proposita had both ocular and skeletal involvement, with unilateral iridal and retinal coloboma, rudimentary 12th ribs and a mild scoliosis. Two other affected relatives had Klippel-Feil and vertebral fusion and one affected relative had scoliosis (Ye et al., 2010; Pedigree #1).

unilateral microphthalmia.[7] To explain the non-penetrance, it has been hypothesized that sequence variations in different BMP ligands can contribute additively to the ocular or skeletal phenotypes.[7] However, we did not find any increased genetic burden in known genes for MAC or in BMP ligands in the proposita compared to her unaffected mother who carries the same sequence alteration. Non-penetrance has previously been demonstrated for genes involved in structural eye defects, such as *OTX2*, *BMP4* and *OPA1*,[21–23] suggesting that deleterious sequence variants in these genes do not lead to consistent phenotypes.

Non-penetrance has also been documented for *GDF6*, another member of the TGF family. In that gene, the sequence variant c.746C > A, predicting p.(Ala249Glu), was present in patients with eye findings, but also in one individual with normal eyes. On two occasions, this mutation was inherited from an unaffected parent.[19,24,25] In a study that examined 11 patients with developmental eye defects using exome sequencing, one patient showed heterozygosity in *GDF3* for c.974C > T, predicting p.(Pro325Leu), a sequence variant that was predicted to be highly conserved and damaging, but that was nevertheless inherited from the patient's unaffected father. The child with p.(Pro325Leu) had microphthalmia and coloboma, but was also a heterozygote for two missense mutations in *CYP1B1*. Both genes were considered relevant to pathogenesis of the eye defects in view of the role of *CYP1B1* in retinoic acid synthesis that regulates choroid fissure closure.[2] Similarly, a child with horizontal and rotatory nystagmus, bilateral iris colobomas with severe colobomatous microphthalmia, bilateral foveal hypoplasia, abnormally small optic discs with reduced optic nerve diameters and severely compromised vision (20/200) had p.Arg266Cys in *GDF3*, the same mutation as our proband, together with p.Ala199Thr in *GDF6*, whereas her parent carrying only the *GDF3* p.Arg266Cys variant had mild bilateral iris colobomata and microphthalmia, normal optic discs and mildly affected vision (20/40) in each eye.[7] As transfection of mutant *GDF3* containing p.Arg266Cys together with *GDF6* containing p.Ala199Thr resulted in greater reduction of *SOX9* activation compared to co-transfection with a construct containing either sequence variant alone, this suggests that these two sequence variants may have additive effects.[7] We did not find any clear candidate genes for the eye defects that would interact with *GDF3* in our proposita, but novel genes may still be important in this patient and her extended family. In addition, there are no animal

models of loss of function for *Gdf3* that have had eye defects. In the mouse, *Gdf3* homozygous null embryos have early morphological defects, with aberrant anterior visceral endoderm induction and migration, processes that are regulated by both BMP and Nodal signaling, although neither pathway has been studied in the context of the null mice.[16] These early developmental defects have precluded study of later developmental roles for this gene and conditional knock-outs have not yet been generated. The *Danio rerio* orthologue of *gdf3*/*gdf1*, namely *dvr1*[7] has also been studied. *In-situ* hybridization showed modest expression of *dvr1* in the head region at 14–18 hours post fertilization (hpf) and injections with antisense morpholinos (MOs) targeting the translation start site of *gdf3* recapitulated the human ocular phenotypes with reduced eye size, colobomas, and defective development of the lens and retina, including retention of nuclei in the lens and shortening or missing photoreceptor outer segments.[7] Injection of a translational MO targeting *gdf1*/*gdf3* also resulted in misexpression of the ventral retinal patterning marker, *foxg1*, suggesting a role in eye formation. However, these studies could have been confounded by decreased *gdf1* expression in addition to reduced *gdf3* expression.[7]

Lastly, functional studies have been performed for the sequence variants in *GDF3* and do provide some evidence in favor of pathogenicity. In addition to the reduced levels of mature GDF3 seen with p.Arg266Cys, western blot analysis of separately transfected COS-7 cell lysates detected mildly reduced amounts of full-length p.Leu305Pro-mutant protein and p.Arg195Gln-mutant protein in the cytosol, with striking reductions in the amount of mature p.Leu305Pro protein and p.Arg195Gln protein in the media.[7] The p.Arg274Trp mutation changed a conserved hydrophilic residue to a hydrophobic residue and was not present in 480 control DNA samples.

4. Conclusions

The sequence variant p.Arg266Cys in *GDF3* has previously been reported as a causative mutation for eye defects. However, in view of the non-penetrance observed with this variant and the lack of direct evidence from animal models for the involvement of *Gdf3* loss of function in eye defects, we consider it plausible that p.Arg266Cys and other *GDF3* sequence variants may act as modifiers, rather than causative genes, in combination with other genetic and possibly environmental factors. A greater number of reported variants and phenotypic analysis are still needed to classify this gene and the p.Arg266Cys variant.

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Authorship

All authors attest that they meet the current ICMJE criteria for Authorship.

Conflict of interest

The following authors have no financial disclosures: TB, MK, DW, RL, PT, EW, SK, AdS, PK, AS.

Patient consent

Written consent to publish the report was obtained from the patient.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ajoc.2017.06.006.

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