BMP4 loss-of-function mutations in developmental eye disorders including SHORT syndrome

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Abstract BMP4 loss-of-function mutations and deletions have been shown to be associated with ocular, digital, and brain anomalies, but due to the paucity of these reports, the full phenotypic spectrum of human BMP4 mutations is not clear. We screened 133 patients with a variety of ocular disorders for BMP4 coding region mutations or genomic deletions. BMP4 deletions were detected in two patients: a patient affected with SHORT syndrome and a patient with anterior segment anomalies along with craniofacial dysmorphism and cognitive impairment. In addition to this, three intragenic BMP4 mutations were identified. A patient with anophthalmia, microphthalmia with sclerocornea, right-sided diaphragmatic hernia, and hydrocephalus was found to have a c.592C>T (p.R198X) nonsense mutation in BMP4. A frameshift mutation, c.171dupC (p.E58RfsX17), was identified in two half-siblings with anophthalmia/microphthalmia, discordant developmental delay/postaxial polydactyly, and poor growth as well as their unaffected mother; one affected sibling carried an additional BMP4 mutation in the second allele, c.362A>G (p.H121R). This is the first report indicating a role for BMP4 in SHORT syndrome, Axenfeld–Rieger malformation, growth delay, macrocephaly, and diaphragmatic hernia. These results significantly expand the number of reported loss-of-function mutations, further support the critical role of BMP4 in ocular development, and provide additional evidence of variable expression/non-penetrance of BMP4 mutations.

Introduction

Bone morphogenetic protein 4 (BMP4) is a member of a large cytokine family related to the transforming growth factor-beta proteins. Bmp4 is an important regulator of
normal development causing numerous embryonic defects when mutated or misexpressed in vertebrates (Chang et al. 2001; Furuta and Hogan 1998; Hogan 1996; Jiao et al. 2003; Solnica-Krezel 1999).

In humans, disruption of BMP4 by deletion or mutation was shown to be associated with ocular, digital, and brain anomalies. BMP4 is located at chromosome 14q22–q23, near the OTX2 gene, which is a well-established cause of anophthalmia/microphthalmia (Ragge et al. 2005; Schilter et al. 2010). Deletion of BMP4 was first demonstrated in combination with OTX2 deletion in three patients with anophthalmia, pituitary defects, developmental delay, and structural brain anomalies with syndactyly, brachydactyly, and genitourinary anomalies in one (Bakrania et al. 2008; Nolen et al. 2006). A patient with de novo deletion of BMP4 but not OTX2 was subsequently reported with congenital glaucoma and sclerocornea, postaxial polysyndactyly, brain anomalies, and developmental delay (Hayashi et al. 2008). Two families with anophthalmia/microphthalmia and intragenic mutations in BMP4 have been identified (Bakrania et al. 2008). The first mutation, c.226delAG (p.S76CfsX29), was identified in a proband with unilateral anophthalmia and coloboma, retinal dystrophy, and small anterior segment of the contralateral eye, along with postaxial polydactyly, structural brain anomalies, and learning difficulties. The c.226delAG mutation was seen in three additional family members affected with high myopia and/or polydactyly. The second, c.278A>G (p.E93G), was identified in a proband with bilateral microphthalmia, broad hands with low-placed thumbs, brain anomalies, and developmental delay. The p.E93G mutation was inherited from the father, whose only anomaly was mild inferi or pigmenta tion of both retinas. A third variant in BMP4, c.751C>T (p.H251Y), was detected in a proband with mild microphthalmia (corneal diameter 10.7 mm; axial length 20.2 mm) and anterior segment anomalies as well as his unaffected brother (Zhang et al. 2009). The role of BMP4 in anterior segment development/ glaucoma is further supported by animal studies demonstrating anterior segment dysgenesis and elevated intraocular pressure in Bmp4+/- mice and defects in BMP4 signaling in experimental glaucoma models (Chang et al. 2001; Wordinger et al. 2007).

In addition to ocular phenotypes, BMP4 was shown to be associated with cleft lip and palate (Suzuki et al. 2009), renal malformations (Weber et al. 2008), and colorectal cancer (Lubbe et al. 2011). Heterozygous missense mutations in BMP4 were identified in seven probands with subepithelial, microform, and overt cleft lip/palate, and a nonsense mutation, p.R198X, was identified in another proband with overt cleft lip and palate; no information was provided regarding presence or absence of additional anomalies in these patients. All of the missense mutations were inherited from either mildly affected (three cases) or unaffected parents (four cases); inheritance of the nonsense mutation was not examined (Suzuki et al. 2009). Heterozygous missense mutations in BMP4 were also identified in four probands with renal agenesis, dysplasia, or hypoplasia, and a homozygous missense mutation was seen in one proband with cystic dysplasia of the kidneys; again, no details were provided regarding presence or absence of additional anomalies. Three of the mutations were inherited from unaffected parents (including both parents of the homozygous case): one was de novo, and the parents were not tested in the remaining two cases (Weber et al. 2008). Finally, BMP4 coding region variants were identified in six probands with colorectal cancer in a case/control mutation screen (Lubbe et al. 2011). Based on in silico analysis and control data, the authors classified two missense and one nonsense (p.R286X) variants as pathogenic mutations; no inheritance pattern analysis was performed, and no additional systemic anomalies were documented for any of these cases (Lubbe et al. 2011).

We present results from screening of BMP4 in patients with various ocular conditions. Five new deletions/mutations in BMP4 were found in four families. Our screening identified two deletions of BMP4 but not OTX2, including one in a patient with SHORT syndrome, one nonsense mutation, and one frameshift mutation which was detected in two half-siblings with discordant phenotypes with an additional missense mutation in one.

**Materials and methods**

This human study was approved by the Institutional Review Boards of Children’s Hospital of Wisconsin, the University of Michigan, and Albert Einstein Healthcare Network with written informed consent obtained from every subject. Genomic DNA was extracted using standard procedures from blood or buccal samples. Complete sequence of the BMP4 coding region (reference sequence NM_001202.3) was obtained for 133 patients including 60 with anophthalmia/microphthalmia (34 syndromic), 38 anterior segment disorders (29 syndromic) including 3 with SHORT syndrome, 16 cataracts, 4 coloboma, 5 high myopia, and 10 other disorders using the following primers: set 1 forward, cttgatctttctgacctgct, and reverse, ttcttgaggttaagctgct, PCR product equal 656 bp and set 2 forward, attgccacccagctgct, and reverse, cagctagagagtaacgctgct, PCR product equal 1,069 bp. Patients with a genetic etiology identified in previously reported and unreported screens were excluded. Sequences were reviewed manually and using Mutation Surveyor (Soft-Genetics, State College, PA, USA). All mutations were confirmed by independent sequencing reactions using new
PCR products. Screening for deletions of \textit{BMP4} was performed using Affymetrix Genome-Wide Human SNP Array 6.0 or TaqMan assays in 89 of the above patients, including 40 patients with anophthalmia/microphthalmia and 26 with anterior segment disorders. In addition, all deletions were confirmed using TaqMan assays \cite{data not shown}. The patient was found to have a 2,263-kb deletion (1,508 probes in haploid state; minimum interval chr14: 51,402,258–53,665,008; maximum interval chr14: 51,400,039–53,667,259; based on UCSC 2006 hg18 assembly) of 14q22.1–14q22.2 which deletes one copy of \textit{BMP4} as well as \textit{NGN2}, \textit{NID2}, \textit{C14orf166}, \textit{PTGDR}, \textit{PTGER2}, \textit{TXNDC16}, \textit{GPR137C}, \textit{ERO1L}, \textit{PSMC6}, \textit{STYX}, \textit{GCPNAT1}, \textit{PLEKHC1}, and \textit{DDHD1} (Fig. 2a). \textit{BMP4} is the most distal gene included in the deletion, but the deletion extends past the end of the coding region of \textit{BMP4}. Quantitative PCR data obtained with TaqMan probes confirmed deletion of one copy of \textit{BMP4} (Fig. 2b) and presence of both copies of \textit{OTX2} (data not shown). The mother (noted to have high myopia but otherwise normal ocular and other systemic features) was tested by TaqMan assay and showed no evidence of \textit{BMP4} deletion; the unaffected father is not available for testing.

Patient 2 (Fig. 1d; Table 1) is a 12-year-old Caucasian female with right corectopia, bilateral microphthalmia, bilateral persistence of the pupillary membrane, high myopia, strabismus, and nystagmus. The patient also has a history of hypotonia and mild–moderate cognitive impairment. The patient has dysmorphic facial features including maxillary hypoplasia with midface flattening, thin upper lip, broad nasal bridge and tip, telecanthus, and had a preauricular ear tag on the right. She has normal growth, head circumference, umbilicus, hands, and feet. Head imaging studies are not available. Peripheral blood chromosome analysis showed 46, XX (550 bands), and subtelomeric FISH was normal. The patient is adopted, so information about family history is limited, and no family members are available for testing. The patient was found to have a 158-kb deletion (122 probes in haploid state; minimum interval chr14:53,361,728–53,520,165; maximum interval chr14:53,352,059–53,520,859; based on UCSC 2006 hg18 assembly) involving 14q22.1–14q22.2 which deletes one copy of \textit{BMP4} (Fig. 2a). No other genes are present in the deleted region. Quantitative PCR data obtained with TaqMan probes confirmed deletion of one copy of \textit{BMP4} (Fig. 2b) and presence of both copies of \textit{OTX2} (data not shown).

Patient 3 (Fig. 1e; Table 1) is a 19-month-old Caucasian male with right anophthalmia/severe microphthalmia, left mild microphthalmia with sclerocornea, facial asymmetry, and right-sided diaphragmatic hernia. His height and weight were normal at 11 weeks of age (60.4 cm/25th–50th centile and 14.9 kg/50th–90th centile); he is very small with some minimal correction for his vision deficits. He has macrocephaly (43.5 cm at 11 weeks/ 90th–97th centile; 51 cm at 19 months/ >97th centile), a large anterior fontanelle, and hydrocephalus treated with a
subdural-peritoneal shunt. Brain MRI at 4 months of age confirmed the ocular findings and showed macrocrania with very prominent subarachnoid spaces, superimposed overlying subdural collections as well as diffuse cerebral atrophy with ventricular prominence. Clinical chromosomal microarray (using Affymetrix Whole Genome-Human SNP Array 6.0) was normal. He was found to have a heterozygous c.592C>T (p.R198X) mutation, previously reported in cleft lip/palate (Suzuki et al. 2009) and predicted to result in premature termination of the BMP4 protein (Fig. 3a). Neither parent carries the mutation.

Patient 4 (Fig. 1f; Table 1) is a 3.5-year-old Caucasian female with bilateral clinical anophthalmia, small ears, and a small left renal cyst. Her birthweight was 3.35 kg (25th–50th centile), birth length was 49.5 cm (50th centile), and birth head circumference was 34 cm (50th centile). At 36 months, her weight was 11 kg (<3rd centile), and height was 93 cm (25th–50th centile). She has normal development, craniofacial features, hands, and feet. Head CT in the neonatal period showed significantly small globes, minimal ocular tissue, and absent optic nerves but otherwise normal brain structures. She was found to have a heterozygous c.171dupC (p.E58RfsX17) mutation along with a second variant, c.362A>G (p.H121R), in BMP4 (Fig. 3a, b). This variant was found to alter a conserved amino acid (Fig. 3b) and is predicted to be tolerated by SIFT (Ng and Henikoff 2003; http://sift.jcvi.org/) while probably damaging by PolyPhen-2 (Adzhubei et al. 2010; http://genetics.bwh.harvard.edu/pph2/) analysis. Both the p.E58RfsX17 and p.H121R mutations affect exon 2 of BMP4 and were determined to be positioned on different alleles by PCR cloning and DNA sequencing of isolated alleles. The unaffected mother of Patients 4 and 5 was found to carry the same heterozygous c.171dupC (p.E58RfsX17) mutation along with a second variant, c.362A>G (p.H121R), in BMP4 (Fig. 3a, b). This variant was found to alter a conserved amino acid (Fig. 3b) and is predicted to be tolerated by SIFT (Ng and Henikoff 2003; http://sift.jcvi.org/) while probably damaging by PolyPhen-2 (Adzhubei et al. 2010; http://genetics.bwh.harvard.edu/pph2/) analysis. Both the p.E58RfsX17 and p.H121R mutations affect exon 2 of BMP4 and were determined to be positioned on different alleles by PCR cloning and DNA sequencing of isolated alleles. The unaffected mother of Patients 4 and 5 was found to carry the p.E58RfsX17 mutation with no evidence of mosaicism (data not shown); the mutation apparently occurred de novo since the unaffected maternal grandparent of Patients 4 and 5 was found to carry wild-type BMP4 alleles. The unaffected father of Patient 5 was not available for testing. Neither parent was available for formal ophthalmological examination, so mild ocular anomalies could not be ruled out.

Screening of 179 Caucasian, 89 African American, 91 Asian, and 93 Hispanic control individuals did not identify any of the three coding region mutations discussed above.
| BMP4 mutation | Eye | Digit | Brain/neuro | Craniofacial | Growth | Other | Family history |
|---------------|-----|-------|-------------|--------------|--------|-------|----------------|
| **Deletions**<sup>b</sup> | | | | | | | |
| Patient 1, this study | 2,263-kb deletion (+ 13 genes) | Rieger anomaly, congenital glaucoma microcornea, nystagmus | Hands/feet appear small | Mild gross motor delay with hypotonia; normal brain structures | Macrophtalphy, prominent forehead, sunken eyes, small chin, hypoplastic nares | Height and weight ≤ 3rd centile | SHORT syndrome; hypertexensible joints, teething delay, lipodystrophy, umbilical anomaly | Parents unaffected; mother does not carry deletion; father not tested |
| Patient 2, this study | 158-kb deletion (BMP4 only) | Microphthalmia, persistence of pupillary membrane, high myopia, strabismus, nystagmus, coretopia (right) | WNL | Mild-moderate cognitive impairment, hypotonia; no imaging studies | Maxillary hypoplasia, midface flattening, thin upper lip, broad nasal bridge and tip, telecanthus, preauricular ear tag | WNL | None | Not available |
| Hayashi et al. (2008) | 2,700-kb deletion (+ 17 genes) | Congenital glaucoma, sclerocornea | Bilateral postaxial polydactyly (feet) | Global delay; Decreased brain white matter, lateral ventricular dilatation | Micrognathia | Weight < 3rd centile | None | De novo deletion |
| **Intragenic mutations** | | | | | | | |
| Patient 3, this study | c.592C>T (p.R198X) | Anophthalmia; microphthalmia with sclerocornea | WNL | Development normal; hydrocephalus, diffuse cerebral atrophy | Facial asymmetry, macrocephaly, large anterior fontanelle | Height > 97th centile | Right sided diaphragmatic hernia, laryngomalacia, inguinal hernia | De novo mutation |
| Patient 4, this study | c.171dupC (p.E58RX17) | Anophthalmia | WNL | Development normal; Normal brain structures | Small ears | Height < 3rd centile | Small renal cyst (left) | Affected half-sister (Patient 5) and unaffected mother carry the mutation |
| Patient 5, this study | c.171dupC (p.E58RX17); c.362A>G (p.H121R) in trans | Anophthalmia (left), blepharophimosis | Bilateral postaxial polydactyly (hands) | Development normal; normal brain structures | Telecanthus, relative macrocephaly (75<sup>th</sup> centile), frontal bossing | Height and weight < 3rd centile | None | Above; Father not available |
| Bakrania et al. (2008) | c.226delAG (p.S76CfsX29) | Anophthalmia; Microanterior segment, iris and chorioretinal coloboma, retinal dystrophy | Bilateral postaxial polydactyly (feet) | Learning difficulties; hypoplastic corpus callosum, enlarged trigones, sulcal widening | WNL | Not reported | None | Mutation seen in mother, grandmother, and great aunt with polydactyly and/or high myopia |
| Suzuki et al. (2009) | c.592C>T (p.R198X) | Not reported | Not reported | Not reported | Cleft lip and palate | Not reported | Not reported | Parents not tested |
| Lubbe et al. (2011) | c.856C>T (p.R286X) | None | None | None | None | None | Not reported | Colorectal cancer diagnosed at 42 years | Parents not tested; no first degree relative with colorectal cancer |

<sup>a</sup> Nucleotide numbering is relative to reference sequence NM_001202.3 where +1 is the A of the ATG initiation codon

<sup>b</sup> Patients with deletion of OTX2 in addition to BMP4 were excluded as OTX2 is a well-established cause of ocular and pituitary defects; therefore, the contribution of BMP4 to the phenotype could not be determined
Discussion

This is the first report of BMP4 deletion in a patient with the complete constellation of features comprising SHORT syndrome (Patient 1). SHORT syndrome was first described in 1975 and is characterized by Short stature, hyperextensibility of joints or inguinal Hernia, ocular depression, Rieger anomaly, and delay in dental eruption (Teeth) (Gorlin et al. 1975; Sensenbrenner et al. 1975). Autosomal dominant inheritance has been suggested (Koenig et al. 2003), but the genetic basis of SHORT syndrome is currently poorly understood. One case report identified a familial translocation, t(1;4)(q31.2;q25), presumably disrupting the PITX2 locus, in a child with SHORT syndrome and his mother with Axenfeld–Rieger syndrome and polycystic ovary syndrome (Karadeniz et al. 2004), but no other studies have replicated involvement of PITX2 in SHORT syndrome, which was also excluded in Patient 1 in this study.

Since the patient with SHORT syndrome (Patient 1) is affected with a deletion of the BMP4 region, we compared the “SHORT” deletion with other patient deletions of this region. Both BMP4 deletions reported in this study are smaller than the previously reported deletion of BMP4 without OTX2 involvement (Hayashi et al. 2008). Patient 2 (reported in this manuscript) with deletion of BMP4 only and the previously reported patient with a 2.7-Mb deletion involving BMP4 (Hayashi et al. 2008) did not demonstrate a SHORT syndrome phenotype. There are seven genes which are deleted in Patient 1 with SHORT syndrome, but not in Patient 2 or the patient presented by Hayashi et al. (2008): GNG2, NID2, C14orf166, PTGDR, PTGER2, C14orf166, PTGDR, PTGER2,
TXNDC16, and GPR137C. Although none of these genes represents an obvious candidate for the observed phenotype based on current data (http://www.ncbi.nlm.nih.gov/omim), SHORT syndrome may be a contiguous gene deletion syndrome which requires deletion of one (or more) of these genes in addition to BMP4.

Analysis of other phenotypic information presented in this manuscript as well as previously reported data further supports the role of BMP4 in SHORT syndrome. The association of BMP4 with anterior segment dysgenesis (Patients 2 and 3; Chang et al. 2001; Hayashi et al. 2008; Zhang et al. 2009), poor growth with height and/or weight measurements below the 3rd percentile (Patients 4 and 5; Hayashi et al. 2008), macrocephaly (Patients 3 and 5) and craniofacial/dental development in human patients (Patient 2; Suzuki et al. 2009) and animal models (Fujimori et al. 2010; Vainio et al. 1993; Zhang et al. 2000) is consistent with features of SHORT syndrome. The fact that other patients with BMP4 deletions/mutations do not demonstrate the full SHORT syndrome phenotype may be explained by variable phenotypic expressivity of BMP4 mutations and modification of their effect(s) by other genetic factors located elsewhere in genome. The observed incomplete penetrance/variable expressivity of BMP4 mutations and their wide phenotypic spectrum are in agreement with this possibility (Bakrania et al. 2008; Lubbe et al. 2011; Suzuki et al. 2009; Weber et al. 2008; Zhang et al. 2009). Screening of additional patients with SHORT syndrome is needed to determine the role/frequency of BMP4 mutations in this phenotype; in our study, one out of three patients diagnosed with this rare condition demonstrated BMP4 deletion.

The BMP4-positive phenotypes reported in this manuscript also show overlap with the Axenfeld–Rieger spectrum (Alward 2000; Rieger 1934, 1935; Shields et al. 1985) caused by mutations in the PITX2 and FOXC1 genes (Semina et al. 1996; Tümer and Bach-Holm 2009). This spectrum is characterized by ocular findings that include posterior embryotoxon, hypoplastic iris, irido-corneal adhesions and glaucoma and additional systemic defects such as craniofacial dysmorphism, dental hypoplasia and redundant periumbilical skin. Patient 1 (SHORT syndrome) has the characteristic Rieger anomaly in combination with atypical dental and umbilical anomalies, Patient 2 shows anterior segment dysgenesis and characteristic craniofacial dysmorphism including maxillary hypoplasia, and Patient 3 demonstrates anterior segment dysgenesis. Taken together with the potential role of both PITX2 (Karadeniz et al. 2004) and BMP4 (this paper) in SHORT syndrome, these data strongly suggest involvement of both factors in the same developmental processes. This is supported by previous studies in animal models which have shown that Bmp4 and Pitx2 act in a common pathway in craniofacial/dental and left–right asymmetry...
development (Liu et al. 2003; Lu et al. 1999; St Amand et al. 2000; Tsiaris and McMahon 2009). Specifically, Pitx2 was shown to be a repressor of Bmp4 expression (Liu et al. 2003; Lu et al. 1999), but Bmp4 was also able to repress Pitx2 expression (St Amand et al. 2000).

The c.171dupC (p.E58RfsX17) mutation identified in Patients 4 and 5 represents the most 5’ nonsense mutation reported to date and is expected to result in a complete loss of function. This allele is likely to result in an absence of protein product due to nonsense-mediated (NMD) decay of the mutant mRNA since the stop codon associated with this mutation is located more than 55 nt (152 nt) from the end of the second to last exon (Holbrook et al. 2004; Khajavi et al. 2006). If present, the mutated protein is predicted to be 14% of its normal length and lack the transforming growth factor beta (TGFβ)-like domain and 91% of the TGFβ propeptide.

Our results further support the variable expressivity/incomplete penetrance of BMP4 mutations, as has been shown in previous publications (Bakrania et al. 2008; Suzuki et al. 2009; Weber et al. 2008; Zhang et al. 2009). While one previous family demonstrated a highly variable phenotype in association with a frameshift mutation (Bakrania et al. 2008), this is the first report of a loss-of-function mutation in an apparently unaffected parent (mother of Patients 4 and 5). In addition, only one patient in our group demonstrated digit anomalies and only one had a brain anomaly, both commonly reported in previously described cases with deletion/nonsense mutations. At the same time, new syndromes/features not described in earlier reports, such as SHORT syndrome, poor growth, macrocephaly, hydrocephalus, and diaphragmatic hernia, were identified. In addition, the nonsense mutation p.R198X, previously seen in a patient with cleft lip and palate and no details about additional features (Suzuki et al. 2009), was also seen in Patient 3 without cleft lip/palate in our study. None of our patients or the previously reported ocular cases demonstrated cleft lip/palate or family history of colorectal cancer, which have been previously reported in association with syndromic ocular phenotypes. Each of the reported missense mutations were not seen in control individuals and several modify conserved amino acids, in more than half of the cases in which family members were tested, the missense mutation was seen in an unaffected relative (8 of 13). This suggests the possibility that some of these missense variants represent rare polymorphisms rather than pathogenic mutations. At the same time, the number of rare BMP4 variants seen in patients with variable phenotypes may suggest a contributory/sensitizing role for BMP4 missense mutations leading to different phenotypes depending on other genetic variants/mutations present in the affected individuals. Additional mutational screens and identification of factors that may be involved in modification of the phenotypic expression of BMP4 mutations are needed to provide insight into the complexity of human phenotypes associated with BMP4 genotypes.

The results of this study confirm the role of BMP4 in developmental ocular anomalies, particularly anophthalmia/microphthalmia and anterior segment defects, and suggest that limb and brain anomalies may not be informative for determining which patients will benefit from molecular screening. Further screening for BMP4 mutations/deletions in patients with SHORT syndrome and a variety of ocular disorders will be important to further...
define the range of anomalies associated with mutations in/ deletions of this gene.

**Ethical standards** All experiments performed comply with the current laws of the United States of America.

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**Conflict of interest** The authors declare that they have no conflict of interest.

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