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SHORT REPORT

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Expanding the genotypic spectrum of *TXNL4A* variants in Burn-McKeown syndrome

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Abstract

The developmental disorder Burn-McKeown Syndrome (BMKS) is characterised by choanal atresia and specific craniofacial features. BMKS is caused by biallelic variants in the pre-messenger RNA splicing factor *TXNL4A*. Most patients have a loss-of-function variant in *trans* with a 34-base pair (bp) deletion (type 1 Δ34) in the promoter region. Here, we identified two patients with BMKS. One individual has a *TXNL4A* c.93_94delCC, p.His32Argfs *21 variant combined with a type 1 Δ34 promoter deletion. The other has an intronic *TXNL4A* splice site variant (c.258-3C>G) and a type 1 Δ34 promoter deletion. We show the c.258-3C>G variant and a previously reported c.258-2A>G variant, cause skipping of the final exon of *TXNL4A* in a minigene splicing assay. Furthermore, we identify putative transcription factor binding sites within the 56 bp of the *TXNL4A* promoter affected by the type 1 and type 2 Δ34 and use dual luciferase assays to identify a 22 bp repeated motif essential for *TXNL4A* expression within this promoter region. We propose that additional variants affecting critical transcription factor binding nucleotides within the 22 bp repeated motif could be relevant to BMKS aetiology. Finally, our data emphasises the need to analyse the non-coding sequence in individuals where a single likely pathogenic coding variant is identified in an autosomal recessive disorder consistent with the clinical presentation.

KEYWORDS
Burn-McKeown syndrome, choanal atresia, craniofacial abnormalities, *DIM1*, non-coding variant, promoter, RNA splicing, spliceosome, *TXNL4A*

1 | INTRODUCTION

Burn-McKeown Syndrome (BMKS, MIM 608572) is an autosomal recessive developmental craniofacial disorder with fewer than 20 families being described in the literature. Although there is clinical overlap with other craniofacial disorders including Treacher Collins syndrome, the recessive mode of inheritance and characteristic constellation of features differentiate BMKS from other craniofacial disorders. Affected individuals present with choanal atresia/stenosis, short palpebral fissures, lower eyelid coloboma, prominent nasal bridge, cleft lip and/or palate and large protruding ears.[1–7] Choanal atresia/stenosis has been reported in all affected individuals to date. Extra-craniofacial phenotypes of conductive and sensorineural hearing loss, congenital heart defects, inguinal hernias and short stature are observed in some patients. One BMKS individual has been reported with intellectual disability and developmental delay.[8]

Wieczorek et al. identified biallelic variants in *TXNL4A* as causative in BMKS.[4] Most affected individuals carry a 34-base pair (bp) deletion (chr18: g.77748581_77,748614del [GRCh37, hg19]), known as the type 1 Δ34) in the *TXNL4A* promoter of one allele combined with a loss-of-function variant on the other allele. Loss-of-function variants include microdeletions, splice site, nonsense and frameshift variants.[4,6] Alternatively, some affected individuals are homozygous for a different 34 bp deletion, (chr18: g.77748604_77,748 637 [GRCh37, hg19], known as the type 2 Δ34) in the *TXNL4A* promoter.[4,6,7] It is proposed that reduced *TXNL4A* expression causes BMKS, with complete loss-of-function likely embryonically lethal.

TXNL4A/DIM1 is a spliceosomal U5 small nuclear ribonucleoprotein particle (snRNP) component, responsible for all precursor mRNA (pre-mRNA) splicing.[9–11] It is postulated that decreased *TXNL4A* expression reduces tri-snRNP assembly disrupting splicing of a specific subset of pre-mRNAs required for craniofacial development.[4,12,13] Mis-splicing of pre-mRNAs relevant to craniofacial development would result in the tissue-specific and restricted phenotype of BMKS patients.

A difficulty hindering the diagnosis of BMKS is the identification of the 34 bp *TXNL4A* promoter deletions from sequencing data. Promoter deletions may not be identified by whole-exome sequencing (WES), while bioinformatics pipelines for whole-genome sequencing (WGS) frequently do not cover non-coding sequences encompassing promoter and deep intronic regions.[14] Here, we identify two unreported individuals with BMKS with novel *TXNL4A* genotypes. We show that a novel *TXNL4A* c.258-3C>G splice acceptor variant in one patient, as well as a previously reported c.258-2A>G variant affecting the adjacent nucleotide, cause skipping of the final exon of *TXNL4A*. Furthermore, we identify potential transcription factor binding sites within the *TXNL4A* type 1 and type 2 Δ34 promoter deletions and use a dual luciferase assay to identify a 22 bp repeated motif which is crucial for *TXNL4A* promoter activity. These findings expand the genetic spectrum of *TXNL4A* variants underlying BMKS and identify why *TXNL4A* Δ34 promoter deletions influence *TXNL4A* expression.

2 | MATERIALS AND METHODS

See Data S1.

3 | RESULTS AND DISCUSSION

3.1 | Identification of novel patients with BMKS from WGS data

We sought to identify undiagnosed patients with BMKS using WGS data from the 100 000 (100 K) Genomes Project. Using available sequence variant data from the standard variant filtering pipeline, we identified heterozygous loss-of-function variants in *TXNL4A* in two individuals with phenotypes consistent with BMKS. Sequence variant filtering identified only *TXNL4A* mono-allelic coding variants meaning the potential diagnoses of BMKS had not been made. We then used manual bioinformatics analysis of WGS data to screen for *TXNL4A* promoter deletions in affected individuals. Both patients were found

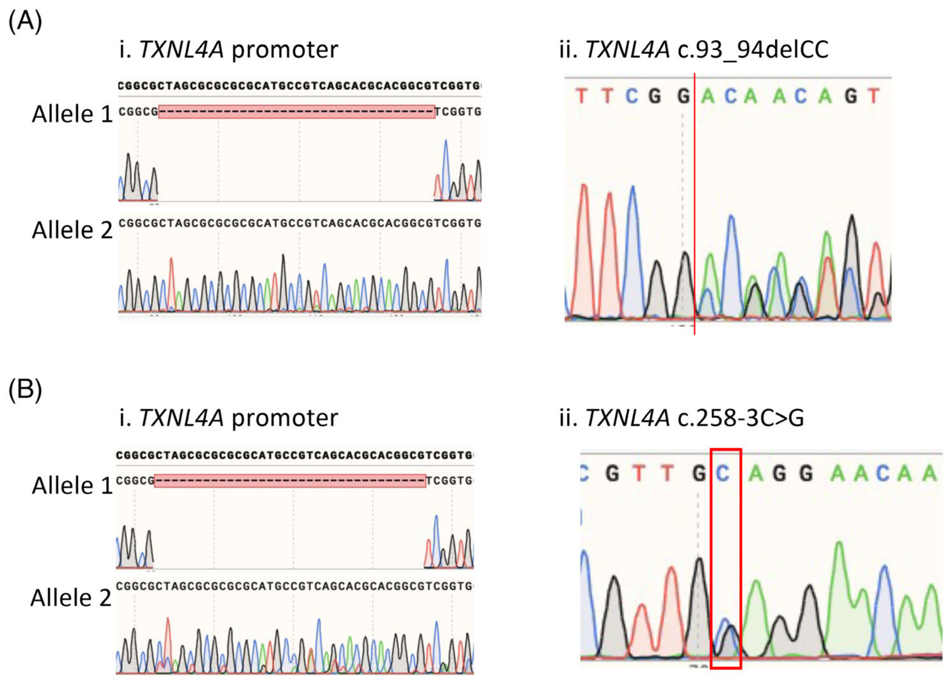

FIGURE 1 Confirmation of biallelic variants in *TXNL4A* from patients with BMKS identified from whole-genome sequencing data. (A) i. heterozygous type 1 34 bp deletion and ii. heterozygous *TXNL4A* c.93_94delCC variants in family 1 proband. Red line indicates position of 2 bp deletion, after which a double sequencing trace indicates heterozygous frameshift. (B) i. heterozygous type 1 34 bp deletion and ii. heterozygous *TXNL4A* c.258-3C>G variant in family 2 proband. Red box indicates single nucleotide variant, with double peak indicating variant heterozygosity [Colour figure can be viewed at wileyonlinelibrary.com]

to have a heterozygous type 1 Δ34 promoter deletion, which was confirmed by Sanger sequencing (Figure 1).

3.2 | Proband phenotyping

The family 1 proband is a white British female only child born to unrelated parents who presented in the genetic clinic in adulthood with mixed conductive sensorineural hearing loss and jaw ankylosis (Figure 2A). She had previous treatment for bilateral choanal atresia and displayed dysmorphic craniofacial features including lower eyelid coloboma, malar flattening, a high palate and micrognathia, right-sided microtia and protruding ears (Figure 2B, Table S1). Sequencing revealed a heterozygous chr18:77748298TGG>T (GRCh37), *TXNL4A* c.93_94delCC (NM_006701), p.His32Argfs*21 (NP_006692) variant with a heterozygous type 1 Δ34 promoter deletion (Table S1). The frameshift variant is not present in gnomAD and has not been previously associated with BMKS. Parental genotyping revealed that the type 1 Δ34 promoter was maternally inherited, while the c.93_94delCC was paternally inherited (Figure 2A, Table S1). The mother is clinically unaffected. The father died at 44 years of oesophageal carcinoma. He possibly had choanal atresia as, at 11–12 years, he had an operation to drill one side out of his nose as his nasal passages had not fully developed. He also possibly had a flat malar region (Table S1). It is possible that the father may be mildly clinically affected based on his reported phenotype. Sanger sequencing of the whole *TXNL4A* coding and promoter sequence for the father did not reveal any additional variants which could account for his craniofacial features. As he was deceased, the father was not recruited to the 100 K Genomes Project. Therefore, WGS was not available. It is unlikely that the oesophageal carcinoma is related to his *TXNL4A* genotype as this association has not been described in other carriers of *TNXL4A* variants. While somatic mutations in some core spliceosome components have been associated with cancer, there are no reports to date of *TXNL4A* mutations in tumours.[15,16]

The family 2 proband is a white British male and only child of healthy, non-related, parents with phenotypic features including choanal atresia, conductive hearing impairment, a cleft upper lip and distinctive craniofacial features including downslanted palpebral fissures, malar flattening and dysplastic ears (Figure 2A). The left ear was atretic with closure of the external auditory ear canal (microtia) (Figure 2C, Table S1). Sequencing revealed a heterozygous chr18:77733859G>C (GRCh37), *TXNL4A* c.258-3C>G (NM_006701) splice acceptor variant and a heterozygous type 1 Δ34 promoter deletion (Table S1). The splice acceptor variant is not observed in the gnomAD population database and has not been previously described in a BMKS patient. However, a variant in the adjacent nucleotide, *TXNL4A* c.258-2A>G (NM_006701) has been described in an individual with BMKS.[6] *In silico* prediction of variant pathogenicity suggested both splice site variants are disease-causing by disrupting the splice acceptor site (Table S1). We conducted minigene splicing assays for the c.258-2A>G and c.258-3C>G variants; both led to complete skipping of the *TXNL4A* final exon (Data S1; Figure S1). Deletion of *TXNL4A* exon 3 in *trans* to a type 1 Δ34 has been reported in another BMKS patient.[4] The heterozygous c.258-3C>G splice acceptor variant was maternally inherited while the heterozygous type 1 Δ34 was paternally inherited (Figure 2A, Table S1). Comparison of the clinical features observed in patients here and previously reported patients is provided in Table S1.

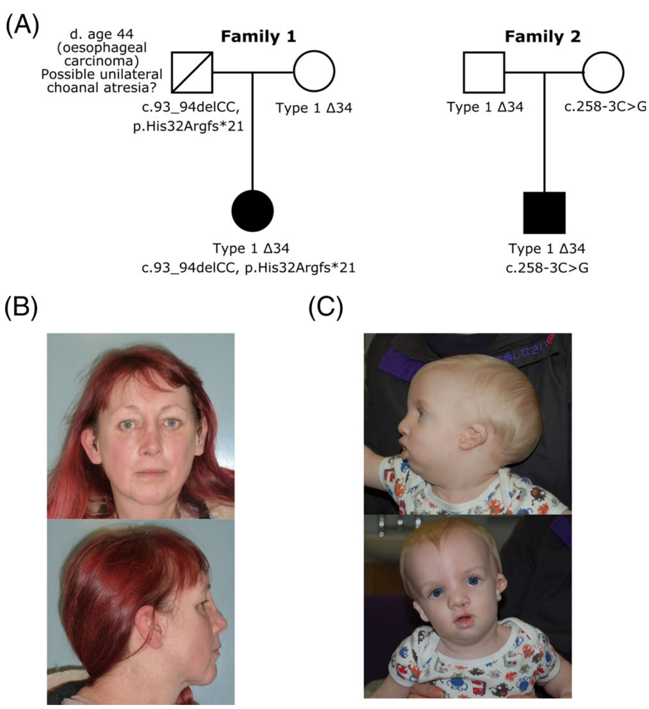

FIGURE 2 Family pedigrees and facial phenotypes of individuals with BMKS. (A) Pedigrees for family 1 and family 2 with *TXNL4A* genotypes indicated. Filled in symbols affected individuals, diagonal line deceased individual. (B) Craniofacial phenotype of affected family 1 individual. (C) Craniofacial phenotype of affected family 2 individual. Consent for publication of photographs was obtained [Colour figure can be viewed at wileyonlinelibrary.com]

3.3 | Identifying putative transcription factor binding sites within the human *TXNL4A* promoter type 1 Δ34

Wieczorek et al. found that *TXNL4A* type 1 and type 2 Δ34 deletions reduced promoter activity by 59% and 72%, respectively.[4] This promoter region consists of two repeated 22 bp motifs separated by a 12 bp spacer, with each Δ34 deletion containing one of the 22 bp repeated motifs with the spacer region overlapping the type 1 and type 2 Δ34 (Figure 3A). These 34 bp regions were proposed to contain binding sites for transcription factors which promote *TXNL4A* expression, the loss of which cause decreased promoter activity in patients and carriers of the deletions.[4] We predicted potential binding sites for four transcription factors (XBP-1, c-JUN, AhR/ARNT and ATF3) in the type 1 Δ34 (Figure 3A). All but three nucleotides in these binding sites were within the

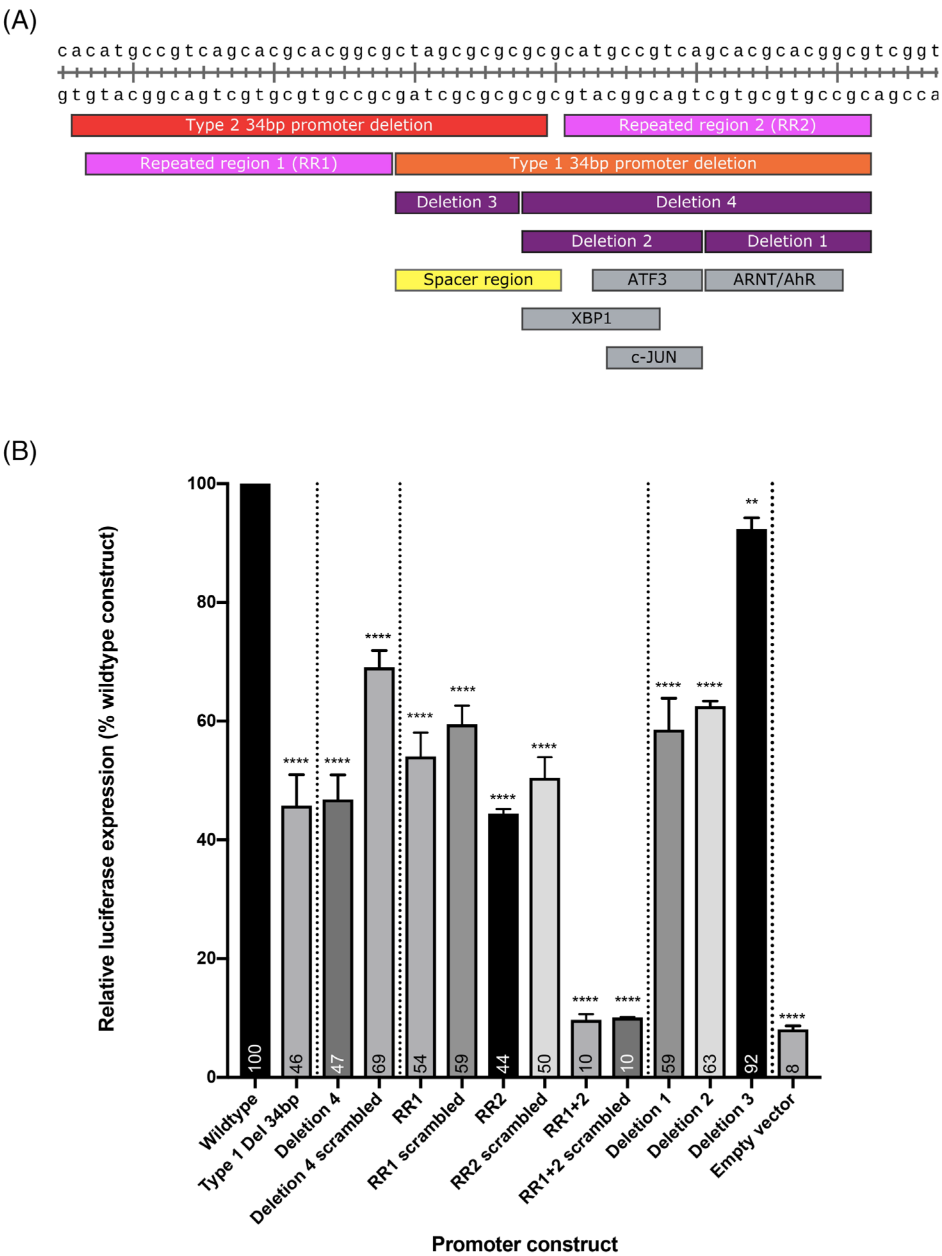

FIGURE 3 Analysis of the human *TXNL4A* promoter. (A) Structure of *TXNL4A* promoter region affected by type 1 (orange) and type 2 (red) 34 bp deletions in BMKS patients; 12 bp spacer region (yellow) and 22 bp repeated regions (pink). Putative transcription factor binding sites identified using ALGGEN PROMO indicated in grey. Hypothetical deletions 1–4 in luciferase reporter gene constructs are highlighted in purple. B) Effects of *TXNL4A* promoter deletions on luciferase expression. Relative firefly luciferase expression for each construct, normalised to *renilla* luciferase expression, is indicated as a percentage of the wild type promoter region expression. $n = 4$. **p-value <0.01, ****p-value <0.0001 [Colour figure can be viewed at wileyonlinelibrary.com]

repeated 22 bp motif rather than the 12 bp spacer, meaning of the four predicted transcription factors, only XBP-1 is not predicted to also bind to the type 2 Δ34. Interestingly, only twelve heterozygous and one homozygous variant in the 56 bp region of the *TXNL4A* promoter were found in the gnomAD database, indicating an important and sequence-specific role in promoter activity (Data S1; Table S2).

3.4 | In vitro analysis of putative transcription factor binding sites on promoter function

To test whether the identified putative transcription factor binding sites are important in *TXNL4A* promoter function, we cloned a 601 bp *TXNL4A* promoter fragment into a luciferase reporter vector and performed dual luciferase assays. Constructs contained the wild type promoter region, the type 1 Δ34 or several smaller deletions (Figure 3A). Similar to Wieczorek et al., we found type 1 Δ34 reduced promoter activity to 46% (Figure 3B).[4] Smaller deletions (deletions 1 and 2) reduced promoter activity to 59% and 63%, respectively, while deletion 3 (12 bp spacer) only reduced promoter activity by 7% (Figure 3B). Deletion 4 (spanning deletions 1 and 2) reduced activity to 47% (Figure 3B). Scrambling deletion 4 reduced activity to 70%, suggesting sequence specificity of this region (Figure 3B). We then deleted the 22 bp repeated motif within the type 1 Δ34 (repeated region 2, RR2) or type 2 Δ34 (repeated region 2, RR2) (Figure 3A). RR1 reduced promoter activity to 54%, while RR2 reduced activity to 45%, the same as the full type 1 Δ34 (Figure 3B). Deleting or scrambling both RR1 and RR2 together reduced promoter activity to 10% (Figure 3B). These findings suggest that RR1 and RR2 contain the critical nucleotides for *TXNL4A* promoter activity and act independently and cumulatively to promote *TXNL4A* expression.

This study has reiterated the power of WGS in diagnosing patients with rare disorders and emphasises the need to consider non-coding regions when analysing WGS data, especially when a single pathogenic coding variant is identified in a disease-associated gene known to cause a recessive condition consistent with the clinical presentation. We have also developed an analysis approach for screening existing and novel promoter variants in a gene of interest. This approach may prove useful for disorders associated with promoter variants where few patients have been identified and where it is unclear whether a single pathogenic variant or spectrum of different promoter variants underlie the phenotype.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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