

A novel non-stop mutation in *MSX1* causing autosomal dominant non-syndromic oligodontia

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Oligodontia, which is the congenital absence of six or more permanent teeth, excluding the third molars, may contribute to masticatory dysfunction, speech alteration, aesthetic problems and malocclusion. *Msh* homeobox 1 (*MSX1*) was the first gene identified as causing non-syndromic oligodontia. In this study, we identified a novel heterozygous non-stop mutation (c.910_911dupTA, p.*304Tyrext*48) in *MSX1* in a Chinese family with autosomal dominant non-syndromic oligodontia. This novel mutation substitutes the stop codon with a tyrosine residue, potentially adding 48 amino acids to the C-terminus of *MSX1*. Further *in vitro* study found that mutant *MSX1* could be expressed but had lost its ability to enter the nucleus. This is the first report indicating that a non-stop mutation in *MSX1* is responsible for oligodontia. This study broadens the mutation spectrum for *MSX1* and provides a new way to clarify the mechanism of *MSX1* in tooth agenesis.

Introduction

Tooth agenesis is the most common dental developmental anomaly in humans, and it may contribute to masticatory dysfunction, speech alteration, aesthetic problems and malocclusion (1). The prevalence of dental agenesis of permanent teeth ranges from 2.2% to 10.1% in the general population, excluding the third molars (2). Tooth agenesis is classified into three categories: hypodontia, the absence of one to five permanent teeth, excluding the third molars; oligodontia, the absence of more than five permanent teeth, excluding the third molars; and anodontia, the absence of all teeth. Tooth agenesis may present as part of a syndrome; however, the non-syndromic form is more common. In the majority of cases, tooth agenesis has a genetic basis, although tooth agenesis may also be caused occasionally by environmental factors (e.g. by trauma, chemotherapy and radiation therapy and use of thalidomide during pregnancy) (1).

To the best of our knowledge, non-syndromic tooth agenesis has been associated with mutations or polymorphisms in *EDA*, *AXIN2*, *MSX1*, *PAX9*, *WNT10A*, *EDAR* and *EDARADD* (3–12). The first gene identified as causing non-syndromic tooth agenesis was the transcription factor gene *MSX1* (13). *MSX1* belongs

to a family of transcription factors that are expressed in overlapping patterns at multiple sites of tissue interactions during vertebrate development (14). *MSX1* co-determines the position and shape of teeth, which involves the so-called homeobox code model, linking patterning of tooth types to spatially restricted expression of homeobox genes in the dental mesenchyme. Mice lacking the *MSX1* protein function show a cleft palate, deficient mandibular and maxillary alveolar bones and failure of tooth development (15). Previous reports identified different *MSX1* defects that could cause non-syndromic hypodontia or oligodontia (5,16–21). Most of these *MSX1* mutations responsible for tooth agenesis are missense or nonsense mutations.

Here, we identified a novel non-stop *MSX1* mutation (c.910_911dupTA, p.*304Tyrext*48) in a family with autosomal dominant non-syndromic oligodontia.

Materials and methods

Patient and control samples

The 35-year-old Chinese woman was referred to the Department of Prosthodontics in Peking University Hospital of Stomatology to restore her congenital missing teeth. A panoramic radiograph was taken to verify the exact number of missing teeth. Her family members were subsequently recruited. Ninety-six normal volunteers were selected as control individuals. The Institutional Review Board of Peking University School and Hospital of Stomatology approved this study. Informed consent was obtained from all participants, including the patient's family and the normal controls.

Mutation detection

Genomic DNA of the patient and her family members was isolated from peripheral blood lymphocytes as previously described (22). Four exons of *PAX9*, 2 exons of *MSX1* and 10 exons of *AXIN2*, as well as their exon–intron boundaries, were amplified by polymerase chain reaction according to the methods of Kim *et al.* (19), Lammi *et al.* (23) and Liang *et al.* (24) with modifications provided in supplementary file. Another pair of primers, *MSX1*x2bF (5'-AGCTGGAGAAGCTGAAGATG-3') and *MSX1*x2bR (5'-CATGGCCTCTAGCTCTGTTC-3'), was specifically designed to detect mutations. Once a mutation was detected, the PCR products harboring this mutation were cloned into pGEM-T Easy Vectors (Tiangen, Beijing, China) to determine the exact status of mutation.

Construction of expression plasmids and site-directed mutagenesis

The human *MSX1* cDNA (accession number: NM_002448; Origene, Rockville, MD, USA) was cloned into pcDNA3.1 expression vector (Invitrogen, Grand Island, NY, USA) and pcDNA3.1-*MSX1* was generated, incorporating a Myc epitope tag at the C-terminus of *MSX1*. *In vitro* site-directed mutagenesis using a QuikChange mutagenesis kit (Stratagene, La Jolla, CA, USA) was employed to generate the STOP304Tyrext*48 mutation using the following primers: forward—5'-CTACAGCATGTACCACCTGACATATAGAGGGTCCAG-GTCGCCACCTGTGG-3' and reverse—5'-CCACAGGTGGGCGACCTG-GAACCCTCTATATGTCAGGTGGTACATGCTGTAG-3', which inserted two nucleotides and mutated the SNP rs8670 site. The Myc epitope was also in frame with the amino terminus of mutant *MSX1*.

Cell culture, transient transfection and western blot analysis

COS7 cell were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum and 2 mM L-glutamine in the presence of 5% CO₂. Transient transfection was performed using Lipofectamine 2000 (Invitrogen). Proteins were harvested 48 h after transfection, as previously described (25). The proteins subjected to 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis, transferred to a polyvinylidene fluoride (PVDF) membrane, and incubated with anti-Myc-tag and anti-β-actin rabbit polyclonal

antibodies (Cell Signaling, Danvers, MA, USA). The PVDF membrane was washed and then incubated with peroxidase-conjugate goat anti-rabbit secondary antibodies (Origene, Rockville, MD, USA).

Immunolocalisation

COS7 cells were transiently transfected with pcDNA3.1 expression plasmids containing Myc-tagged wild-type and mutant *MSX1* cDNAs. At 48 h post-transfection, the cells were fixed with 95% ethanol and permeabilised with 0.1% Triton X-100. They were then stained with anti-Myc-tag antibody (Cell Signaling), followed by incubation with Alexa-647-labelled anti-rabbit IgG. The cells were mounted in mounting medium with diamidino-2-phenylindole and photographed with the confocal microscope LSM 510 Meta (Zeiss, Oberkochen, Germany) with a $\times 40/1.00$ numerical aperture oil objective lens.

Secondary structural analysis

PsiPred 3.3 (<http://bioinf.cs.ucl.ac.uk/psipred>) (26) was used to predict the secondary structure of wild-type *MSX1* and elongated mutant *MSX1* (p.*304Tyrext*48).

Results

Clinical details

Prosthodontists at the Peking University hospital of Stomatology performed oral examinations of all participants. The proband was a 35-year-old female in good health. Clinical and radiographic examinations revealed that, in addition to retained deciduous teeth, the patient was missing 19 permanent teeth, including the third molar (Figure 1B and C). The mother

of the proband had normal dentition. The father of the proband was 72 years old and was in complete edentulous status. However, he stated that he and his mother had a history of tooth agenesis (pedigree shown in Figure 1A). All family members denied history of heat intolerance, and their facial features, hair, skin and nails appeared normal, which did not suggest the presence of any systemic disorder.

Mutation analysis

Mutation analysis of *AXIN2*, *MSX1* and *PAX9* was conducted in the proband. A novel non-stop heterozygous mutation (c.910_911dupTA, p.*304Tyrext*48) in the stop codon of *MSX1* gene was identified. We then genotyped all available members of the extended family and found this mutation co-segregated with the disease phenotype, and the mutation was not detected in unaffected family members and 96 normal controls. This heterozygous mutation, induced by the c.910_911dupTA insertion, disrupts the original stop codon (Figure 2A). The non-stop mutation was predicted to result in an open reading frame containing 48 additional amino acids at the C-terminus, resulting in a mutant transcription factor with 351 amino acids, compared with the wild-type *MSX1* protein with 303 amino acids (Figure 2B).

Non-stop mutant *MSX1* (p.*304Tyrext*48) could be expressed in COS7 cells

To test whether the non-stop mutation (c.910_911dupTA, p.*304Tyrext*48) in *MSX1* could be expressed *in vitro*, the wild-type and mutant *MSX1* with a Myc-tag were separately transfected into COS7 cells and detected using an anti-Myc antibody. Western blotting results showed that the wild-type *MSX1* was expressed, whereas an enlarged protein could also be detected in the cells transfected with the mutant *MSX1* plasmids (Figure 3A). The result indicated that the elongated protein created by the non-stop mutation could be expressed *in vitro*.

Non-stop mutation (c.910_911dupTA, p.*304Tyrext*48) affected subcellular localisation of *MSX1* protein

To establish whether the non-stop mutation (c.910_911dupTA, p.*304Tyrext*48) affected the nuclear localisation of the transcription factor *MSX1*, resulting in, or contributing to, the tooth agenesis phenotypes, the subcellular localisation of the non-stop mutation protein was determined by immunofluorescence. The *MSX1* protein with the non-stop mutation (c.910_911dupTA, p.*304Tyrext*48) was localised in the entire cytoplasm, whereas the wild-type *MSX1* protein was located exclusively in the nucleus (Figure 3B).

Non-stop mutation (c.910_911dupTA, p.*304Tyrext*48) changes the structure of *MSX1* protein

Secondary structural analysis showed that the wild-type *MSX1* protein is composed of five α -helices (data not shown). The elongated mutant *MSX1*, however, is composed of six α -helices (Figure 2C). The extended 48 additional amino acid residues in C-terminal sequence generate a new α -helix, finally leading to the change of the structure of *MSX1*.

Discussion

In this study, we identified a novel non-stop mutation in *MSX1* in a Chinese family with autosomal dominant non-syndromic

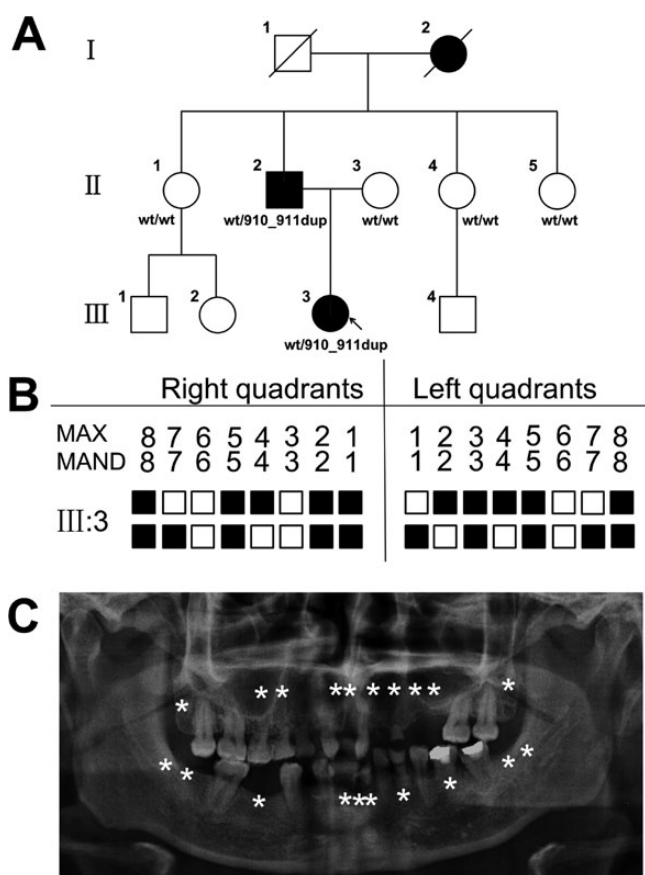


Fig. 1. Pedigree structure and phenotype patterns. (A) Pedigree of the affected family with non-syndromic oligodontia. An arrow indicates the proband. The pedigree displays an autosomal dominant mode of inheritance. (B) Panoramic radiograph of the proband (III3). Stars indicate congenitally missing permanent teeth. (C) Schematic presentation of congenitally missing permanent teeth of the proband (III3).

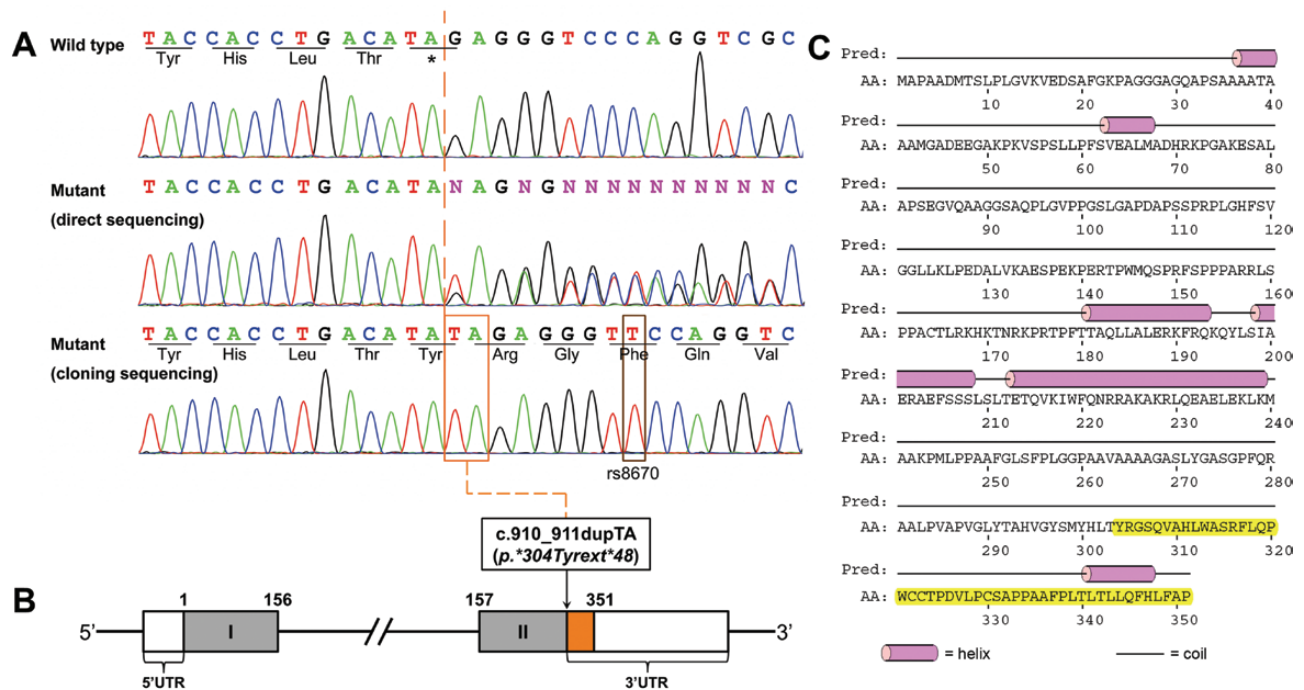


Fig. 2. Detection of MSX1 mutation. (A) Direct sequencing and cloning sequencing of exon 2 in MSX1 of the proband (III3). There are two nucleotide duplications in the stop codon, resulting in a non-stop mutation [TAG(stop) to TAT(Tyr)]. All the affected family members had the same heterozygous c.910_911dup mutation. The duplicated 910_911TA is labelled with an orange frame. SNP rs8670 is labelled with a brown frame. (B) Schematic diagram of the MSX1 gene. Boxes represent exons [white: 5'UTR and 3'UTR; grey: coding sequence, orange: predicted introduced new open reading frame of 144 additional nucleotides (48 addition amino acids) in 3'UTR caused by the non-stop mutation]. (C) The predicted secondary structure of elongated mutant MSX1 protein. The mutant MSX1 protein is composed of six α -helices (pink cylinders), compared with the wild-type MSX1 protein composed of 5 α -helices (data not shown). Coils are presented as straight lines. Forty-eight addition amino acids residues in C-terminal highlighted in yellow generate a new α -helix.

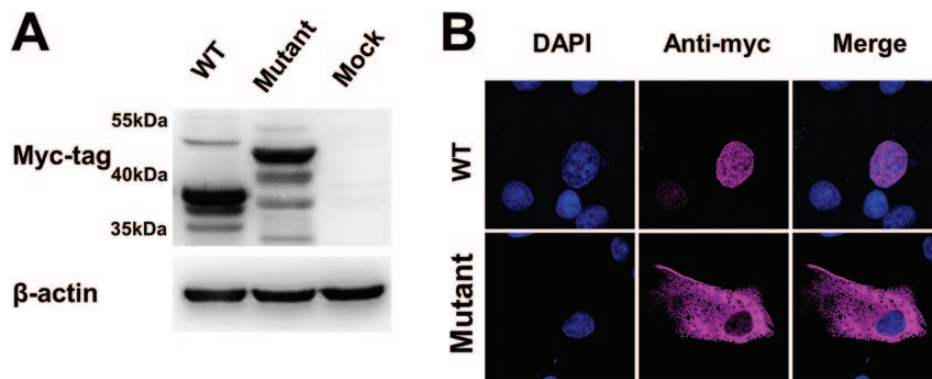


Fig. 3. Expression and subcellular localisation of the wild-type and mutant MSX1. (A) Western blot analyses of cell total protein from wild-type MSX1 or mutant MSX1-transfected COS7 cells using anti-Myc and β -actin antibodies. The elongated mutant protein could be detected. (B) Blue shows artificial colouring of nuclei by diamidino-2-phenylindole; pink shows anti-Myc tag staining. Cells transfected with MSX1-WT showed the localisation of protein in the nucleus. However, the mutant MSX1[non-stop mutation (p.*304Tyrext*48)] was distributed over the entire cytoplasm.

oligodontia. The heterozygous mutation induced by the c.910_911dupTA insertion disrupted the original stop codon, resulting in a non-stop mutation in the normal stop codon 304 (*304Tyr). The non-stop mutation would result in an open reading frame of 351 amino acids compared with 303 amino acids in the wild-type MSX1 protein.

To date, 10 mutations responsible for non-syndromic tooth agenesis have been identified in the MSX1 gene (5,16–20,27–30). Most of them are missense mutations that change only one amino acid in the highly conserved homeodomain sequence (amino acids 167–225). Most of these mutations have been described in families with severe hypodontia inherited in an autosomal dominant fashion. In this study, we found an

interesting non-stop mutation in a Chinese family with non-syndromic oligodontia with autosomal dominant inheritance. The summary of phenotypes associated with mutations in MSX1 shows that patients with mutations in MSX1 are more likely to have agenesis in the second premolar, maxillary first premolar and third molar (1,19). In this study, the proband lacked all of four second premolars, two maxillary first premolars and four third molars. This phenotype conformed to the typical pattern for MSX1 mutations.

To the best of our knowledge, this is the first report of a non-stop mutation associated with tooth agenesis. 'Non-stop' mutations are nucleotide substitutions, insertions or deletions that occur within translational termination (stop) codons and

that can lead to the continued and inappropriate translation of the mRNA into the 3'UTR. This kind of mutation is very rare. Up to 2008, there were >60000 missense and nonsense mutations (in nearly 4000 different genes) listed in the Human Gene Mutation Database that are known to cause, or to be associated with, human inherited disease; only 119 mutations (in 87 different genes) occurred within stop codons (31).

For a non-stop mutation, the normal open reading frame is extended until the next in-frame stop codon is encountered; however, too few human non-stop mutations have been characterised to allow any general conclusions to be drawn as to their likely phenotypic consequences, at either the mRNA or the protein level (31). In our study, the frameshift mutation created a new stop codon with 144 nucleotides downstream of the normal stop codon. Western blotting showed that the mutant protein (p.*304Tyrext*48) could be expressed *in vitro*. Interestingly, the non-stop mutation (p.*304Tyrext*48) in *MSX1* could be translated into protein, but still causes a clinical phenotype. It's possible that this non-stop mutation changed the function of *MSX1*.

The following immunolocalisation confirmed the hypotheses. We found that the non-stop *MSX1* was only detected in the cytoplasm and did not enter the cell nucleus, whereas the wild-type *MSX1* protein was located in the nucleus. Wang *et al.* (32) suggested that nuclear localisation is mediated by the homeodomain of *MSX1*. In the c.910_911dupTA non-stop mutation of *MSX1*, the homeodomain was intact. However, the nuclear localisation was disrupted. This suggested that the extra amino acids may change the protein structure of *MSX1* and affect its function. There are two possible explanations for this. UTRs are known to play crucial roles in the post-transcriptional regulation of gene expression, including modulation of the transport of mRNAs out of the nucleus, translation efficiency, subcellular localisation and stability (33). An incomplete 3'UTR may lead to the mis-localisation of *MSX1* mRNA. Another explanation is that the non-stop mutation created a protein with a changed, non-functional conformation, compared with wild-type *MSX1*. Subsequent secondary structural analysis showed that elongated *MSX1* changes the predicted structure of the *MSX1* protein by generating a new α -helix. The mutated protein could not enter the cell nucleus; it could not function as a transcription factor. The non-stop mutation is heterozygous; therefore, an error in subcellular localisation would lead to a reduced dosage of *MSX1* in patients. Some studies have suggested that haploinsufficiency of *MSX1* could cause tooth agenesis (1,34). Therefore, we conclude that this non-stop mutation in *MSX1* causes autosomal dominant non-syndromic oligodontia in our study family. Kimura *et al.* (21) found a truncated mutant *MSX1*(W139*) in a family with non-syndromic hypodontia and demonstrated the error in subcellular localisation of the mutant *MSX1*(W139*) was the cause of tooth agenesis in this family. Although we found different mutations in *MSX1*, we all agreed that the mistake in subcellular localisation of *MSX1* could be the cause of tooth agenesis.

In conclusion, we identified a novel non-stop mutation (c.910_911dupTA, p.*304Tyrext*48) in *MSX1* in a Chinese family with non-syndromic oligodontia. The non-stop was induced by the c.910_911dupTA insertion, which disrupts the original stop codon to create a novel stop codon 48 amino acids downstream in the 3'UTR of *MSX1*. This is the first report of a non-stop mutation in *MSX1* being responsible for oligodontia. Subsequent *in vitro* experiments provided evidence that this non-stop mutation affected the function of *MSX1*. This study

further confirms that *MSX1* mutations are implicated in familial non-syndromic tooth agenesis. However, the precise role of *MSX1* in tooth agenesis remains to be clarified in further studies.

Supplementary data

Supplementary file is available at *Mutagenesis* Online.

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Conflict of interest statement: None declared.

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