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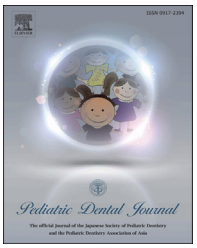

Original Article

Fgf20 and *Fgf4* may contribute to tooth agenesis in epilepsy-like disorder mice

Nao Ogawa [a,*], *Kunihiko Shimizu* [b,c]

[a] *Department of Pediatric Dentistry, Nihon University Graduate School of Dentistry at Matsudo, Matsudo, Chiba, Japan*

[b] *Department of Pediatric Dentistry, Nihon University School of Dentistry at Matsudo, Matsudo, Chiba, Japan*

[c] *Nihon University Research Institute of Oral Science, Chiba, Japan*

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ABSTRACT

Background/purpose: Tooth agenesis is one of the most clearly recognized dental anomalies in the permanent dentition and can be challenging to manage clinically. Recent genetic studies identified several genes related to syndromic and nonsyndromic human dental agenesis. However, the genetic factors related to agenesis of the third molars (M3s), second premolars, and lateral incisors, which are most commonly involved in hypodontia, are still unknown. Therefore, this study aimed to identify the genetic causes of the lacking M3s in epilepsy-like disorder (EL) mice, which have 100% incidence of M3 agenesis.

Methods: M3 tooth germs from EL and C57BL/6 control mice on postnatal day 3 were dissected out and total RNA was extracted. mRNA expressional analysis was carried out using DNA microarray, real-time polymerase chain reaction and *in-situ* hybridization.

Results: DNA microarray analysis revealed significantly decreased expression of *Fgf20* and *Fgf4* and increased expression of EDA in the M3s of EL mice at the bud stage relative to C57BL/6 control mice, which was supported with both reverse-transcriptase polymerase chain reaction and real-time polymerase chain reaction analyses ($p < 0.05$). Furthermore, *in-situ* hybridization revealed low mRNA expression levels of *Fgf20* and *Fgf4* in the M3s of EL mice, whereas strong signals were observed in control mice.

Conclusion: Our results suggest that a decrease of *Fgf20* and *Fgf4* expression may lead to M3 agenesis in EL mice. Understanding the mechanisms controlling tooth agenesis will facilitate the development of strategies for tooth bioengineering.

* *Corresponding author*. Department of Pediatric Dentistry, Nihon University Graduate School of Dentistry at Matsudo, 2-870-1 Sakaecho-Nishi, Matsudo, Chiba 271-8587, Japan.

E-mail address: mana12008@g.nihon-u.ac.jp (N. Ogawa).

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1. Introduction

Tooth agenesis is the most common developmental anomaly of the human dentition. The prevalence of agenesis of the permanent dentition, excluding the third molars (M3s), ranges from 1.6% to 9.6% in the worldwide population [1]. The M3s are missing most frequently, affecting up to 20% of the population, followed by the mandibular second premolars, maxillary lateral incisors, and maxillary second premolars [2,3]. The misalignment, malocclusion, and oral functional problems caused by tooth agenesis in childhood emphasize the importance of understanding the primary causes. Congenital tooth agenesis is characterized by failure of tooth development during tooth organogenesis. More than 150 syndromes are currently known to be related to tooth agenesis [4]. In previous studies, candidate gene mutations in *Msx1* [5], *Pax9* [6], *Axin2* [7], *Wnt10a* [8], *Spry2*, *Spry4* [9], and the ectodermal dysplasia genes EDA [10] have been associated with nonsyndromic tooth agenesis. In most cases of tooth agenesis, the causes remain unknown, indicating that additional genes must be involved [11–13].

Although the causes of M3 or incisor-premolar hypodontia in humans are still unknown, mice have high genetic and chromosomal homology with humans. Thus, isolating the genetic cause of hypodontia in mice may suggest a candidate gene in a homologous region for tooth agenesis in humans. Congenital tooth agenesis is seldom observed in inbred mouse strains: the reported frequency of M3 absence is 18% for CBA/Gr mice [14], 3% for CBA/J mice, and 2% for A/J mice [15]. About 92–100% in mutant stocks such as *tabby* [16], *downless*, and *crinkled* [17] mice affect tooth morphological structure and M3 absence. However, in such mutants, the absence of M3 is part of the pleiotropic phenotypes that are analogous to human hypohidrotic ectodermal dysplasia. Epilepsy-like disorder (EL) mice were established as an animal model for studying epilepsy [18] and evince 100% incidence of M3 agenesis without any generalized craniofacial anomalies [19]. EL mice therefore may be a good model for genetic studies of M3 agenesis or other types of tooth agenesis in humans. Herein, we employed EL mice to identify candidate genes for tooth agenesis.

2. Materials and methods

2.1. Animals

EL mice were obtained from the Laboratory Animal Resource Bank at the National Institute of Biomedical Innovation (Osaka, Japan). C57BL/6 mice were obtained from Japan SLC, Inc. (Shizuoka, Japan). All animals were kept and used according to the guidelines of the Nihon University Intramural Animal Use, Matsudo Chiba, Japan. The experimental protocol was approved by the Nihon University Institutional Animal Experiment Committee (No. AP11MD029).

2.2. Frequency of M3 absence

EL and C57BL/6 mice were killed under deep anesthesia with CO_2 at 8 weeks of age. The heads were soaked in 1% KOH at 42 °C for 24 h, and the soft tissue was removed. The upper and lower M3s were observed under a dissection microscope.

2.3. Histological examination

The heads of EL and C57BL/6 mice were fixed in 10% paraformaldehyde for 24 h, embedded in paraffin, and cut into sections (10 μm). Sections were stained with hematoxylin and eosin. The histopathological changes of M3s on Postnatal Day 3, Postnatal Day 4, and Postnatal Day 5 were observed using light microscopy.

2.4. RNA extraction and total RNA isolation

Development of the M3 tooth germ of EL mice stops in the bud stage on Postnatal Day 3. Ten EL mice and 10 C57BL/6 mice were killed under anesthesia on Postnatal Day 3, and their heads were immediately embedded in Tissue-Tek compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan). Tissue sections (30 μm) for freezing were prepared using a LeicaCM1520 (Leica Microsystems, Wetzlar, Germany). Sections were stained with 0.25% toluidine blue for 10 s. M3 tooth germs were dissected from the upper and lower jaws using a needle under a dissecting microscope, avoiding the tissues surrounding the tooth follicle. A total of 40 M3s from each strain were collected and stored in RNAlater RNA Stabilization Reagent (Qiagen, Tokyo, Japan). Total RNA was extracted from sections with an RNeasy Total RNA kit (Qiagen), according to the manufacturer's instructions. The quantity of RNA was measured using a spectrophotometer (NanoDrop 2000c; Thermo Fisher Scientific, Kanagawa, Japan), and the integrity of the RNA was confirmed with the Agilent 2100 Bioanalyzer (Agilent Technologies, Foster City, CA, USA).

2.5. Microarray hybridization

Microarray hybridization was performed with 14.4 ng total RNA according to the Agilent Expression Array protocols. The RNA was labeled with Cy3 using a Genomic DNA Enzymatic Labeling Kit (Agilent Technologies) and hybridized on the microarray (SurePrint G3 Mouse GE 8 × 60 K Microarray) using an Agilent Gene Expression Hybridization Kit (Agilent Technologies) according to the manufacturer's instructions. After hybridization, the slide glass was washed using Gene Expression Wash Pack (Agilent Technologies), and the images were scanned using an Agilent Microarray Scanner (Agilent Technologies). Fluorescence intensity was calculated using Agilent Feature Extraction software (Agilent Technologies), and data analysis was performed using the GeneSpringGX13 software (Agilent Technologies).

2.6. Reverse-transcription polymerase chain reaction

The DNA microarray results revealed interesting expression patterns for *Fgf20* and *Fgf4*. We also examined EDA, which has been reported to regulate the expressions of *Fgf20* and *Fgf4* in early development. Therefore, we designed primers for reverse-transcription polymerase chain reaction (RT-PCR) analysis of *Fgf20*, *Fgf4*, and EDA (Table 1) based on Ensembl (http://asia.ensembl.org/index.html). Glyceraldehyde-3-