Functional Analysis of Ectodysplasin-A Mutations in X-Linked Non-Syndromic Hypodontia and Possible Involvement of X-Chromosome Inactivation

Yuhua Pan
Southern Medical University
Ting Lu
Southern Medical University
Ling Peng
Southern Medical University
Qi Zeng
Southern Medical University
Xiangyu Huang
Southern Medical University
Xinchen Yao
Southern Medical University
Buling Wu
Southern Medical University
Fu Xiong (xiongfu@smu.edu.cn)
Southern Medical University

Research

Keywords: EDA, Odontogenesis, Dental pulp stem cells, X chromosome inactivation, Tooth agenesis, Odontogenic differentiation

Posted Date: March 6th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-270895/v1

Abstract

Background

Mutations of the Ectodysplasin-A (EDA) gene are generally associated with other developmental anomalies (syndrome hypohidrotic ectodermal dysplasia) or as an isolated condition (non-syndromic tooth agenesis). The influence of *EDA* mutations on dentinogenesis and odontoblast differentiation have not been reported. The aim of the present study was to identify genetic clues for familial nonsyndromic oligodontia and explore the underlying mechanisms, focusing on the role of human dental pulp stem cells (hDPSCs).

Methods

The candidate genes sequences were performed by PCR amplification and Sanger sequencing. Functional analysis and pathogenesis associated with EDA mutations in hDPSCs were also investigated to explore the impact of the identified mutation on this phenotype. Capillary electrophoresis (CE) was used to detect X chromosome inactivation (XCI) on the blood of female carrier.

Results

In this study, we identified a reported EDA mutation in a Chinese family□a missense mutation c.1013C>T (Thr338Met). Transfection of hDPSCs with mutant EDA lentivirus decreased the expression of EDA and dentin sialophosphoprotein (DSPP) compared with those transfected with control EDA lentivirus. Mechanically, the mutant EDA inhibited the activation of the NF-κB pathway. The results of CE showed that symptomatic female carrier had a skewed XCI with a preferential inactivation of the X chromosome carrying the normal allele.

Conclusion

In summary, we demonstrated EDA mutation result in non-syndromic tooth agenesis in heterozygous females and mechanically EDA regulates odontogenesis through the NF-κB signaling pathway in human dental pulp stem cells.

Introduction

Tooth agenesis, the congenital absence of one or more permanent teeth, is the most common abnormality affecting the function and esthetics of patients. Based on the systemic conditions of the patients, congenital tooth agenesis has been divided into non-syndromic hypodontia (NSH) and syndromic hypodontia (SH) [1-3]. Patients with NSH only exhibit tooth-related symptoms (including tooth number abnormality and tooth morphogenesis abnormality). According to the number of missing teeth (not including third molars), tooth agenesis can be classified as hypodontia (less than 6 missing teeth), oligodontia (6 or more than 6 missing teeth), and anodontia (all teeth missing) [4-5]. To date□NSH has been reported to be associated with mutations in ectodysplasin-A (EDA), ectodysplasin-A receptor (EDAR),

EDAR associated death domain (EDARADD), wnt family member 10A (WNT10A), wnt family member 10B (WNT10B), paired box 9 (PAX9), msh homeobox 1 (MSX1), axis inhibition protein 2 (AXIN2) and inhibitor of nuclear factor kappa B kinase regulatory subunit gamma (IKBKG) [6-7]. Among which, EDA mutations could cause SH, which appears as a clinical feature of X-linked hypohidrotic ectodermal dysplasia (XLHED), and also have been linked to isolated tooth agenesis, most likely due to complete or partial disruption of the EDA signaling pathway [8-9].

The EDA gene is located on chromosome Xq13.1 and encodes a protein that belongs the tumor necrosis factor superfamily of ligands. It contains 12 exons, among which there are 8 exons encode two main proteins: EDA1, which binds the EDA-receptor EDAR, and EDA2, a two amino acid shorter variant that binds exclusively to a receptor called XEDAR [10]. The EDA/EDAR/NF-κB pathway has been previously found to be required for normal embryogenesis, particularly in tooth growth and morphogenesis at different stages during tooth development, particularly determining tooth number and contributing to cusp formation [11]. Indeed, previous investigations into the impact of syndrome-causing EDA mutations have shown that most syndrome-causing EDA mutations are predicted to cause an elimination of receptor signaling ultimately. However, some observations have indicated that an EDA mutation in a family with X-linked recessive, non-syndromic tooth agenesis, seems to be found that expression, receptor binding and signaling capability of the mutant EDA1 proteins were only impaired, rather than abolished [12-13].

In our studies, we have found a known EDA mutation c.1013C>T (p.T338M), associated with non-syndromic tooth agenesis, with the mutations located in the TNF homology domain, which was first reported in 2008 [14]. However, the cases reported herein exhibited differences in the clinical phenotype compared with that the previously reported case. In our studies, the heterozygous female carriers only missed a tooth. In the previous studies, the heterozygous female carriers for EDA mutation usually present a highly variable and milder dental phenotype [15-16]. Some researchers have suggested that the dental phenotype variability described in heterozygous female carriers of EDA mutation may occur because of the differential pattern of X-chromosome inactivation (XCI), which still needed further exploration [14,17].

XCI is a random process by which one of the two copies of the X chromosome in female mammals is inactivated [18]. Skewing of X inactivation occurs by chance as a result of this initial inactivation process, by selection as a result of cellular survival or proliferation advantage, or by mutation of the X inactivation apparatus. XCI affects the maternal or paternal X chromosome, followed a ratio of approximately 50:50 in normal females (random XCI) [19-20]. A skewed XCI can cause a female carrier of an X-linked recessive disease to express different protein expression level derived from the mutated allele and experience partial or complete symptoms of the disorder [21-22]. However, the detailed descriptions of X-linked tooth agenesis phenotypes and skewing of X inactivation have been minimal.

In the present study, we identified a known missense mutation of EDA in a Chinese family, which manifested as X-linked recessive NSH, and explored how the mutation in EDA affects the function of

DPSCs. In addition, we investigate genotype and phenotype in patients with X-linked NSH, and we assess a possible relation between severity of clinical symptoms and XCI in female carriers.

Methods

Pedigree and clinical diagnosis

Two Chinese oligodontia patients were identified in the Department of Endodontics at the School of Stomatology, Southern Medical University. Panoramic radiographs confirmed the diagnosis of non-syndromic hypodontia. A pedigree construction was constructed by clinical examinations of available family members and through interviews. A total of 6 family members participated in this study. Tooth agenesis could be traced back to three generations. All subjects gave informed consent and the study was approved by the Ethics Committee of Southern Medical University.

Identification of mutations

To identify disease-associated mutations, we extracted genomic DNA from the peripheral blood of the proband and her family members by a standard phenol/chloroform extraction method. Screening of pathogenic mutations was performed using polymerase chain reaction (PCR) amplification and sequencing the complete exons and exon–intron boundaries of EDA. Details of primers and PCR conditions have been published elsewhere.

Construction of EDA expression vectors, site-directed mutagenesis and their lentivirus package

Full-length human EDA was cloned into the expression vector pcDNA3.1 (Invitrogen), and EDA-Wild Type (WT) was obtained. The c.1013C>T mutant (MUT) was generated using the following primers: forward, 5'-ACACGCAGCATCGAGATGGGCAAGACCAACTAC-3'; and reverse, 5'-GTAGTTGGTCTTGCCCATCTCGATGCTGCGTGT-3', which replaced threonine (Thr) with methionine (Met). Then the plasmid vectors were packaged into lentivirus, which was synthesized by Genechem (Shanghai, China), including control lentivirus (Ctrl), in order to establish a stable transfected cell line in the following studies.

Cell culture and their odontoblastic differentiation

Isolation of human dental pulp stem cells (hDPSCs) was performed as described elsewhere [23]. For odontoblastic differentiation experiments, the cells were cultured in an odontogenic medium (OM), consisting of dulbecco's modified Eagle's medium (DMEM), 10% of fetal bovine serum (FBS), 50mg/mL ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA), 5mM b-glycerophosphate (Sigma-Aldrich), and 10nM dexamethasone (Sigma-Aldrich). The procedure to acquire human tissues was approved by the Ethics Committee of Southern Medical University and Nangfang Hospital of Stomatology.

Cell infection

We overexpressed WT or MUT Flag-EDA in DPSCs with lentivirus following the manufacturer's instructions. The DPSCs were seeded in 12-well plates at a density of 10^5 cells/well and grown for 24h. The cells were infected at a multiplicity of infection (MOI) of 20 in the presence of 5mg/ mL polybrene for 10h at 37°C and 5% CO2.

Western blot analysis

Cells were harvested and then lysed in RIPA buffer (Beyotime, Nanjing, China) supplemented with protease inhibitors. Total protein (20 μg) was separated by 10% SDS-polyacrylamide gel and transferred onto a polyvinylidene difluoride (PVDF) membrane (Amersham, Little Chalfont, UK). The membranes were blocked for 1h with 5% skim milk and incubated overnight at 4°C with anti-FLAG anti-GAPDH (1:1000; proteintech, China), anti-DSPP (1:1000; Santa Cruz Biotechnology), anti-p65,anti-p-p65,anti-IkBa,anti-p-IkBa (1:1000; SAB China) antibodies. The next day, the membranes were incubated for 1h at 37°C with the corresponding secondary antibodies (Proteintech, China), and the immunoreactive proteins were visualized with the ECL Kit (Beyotime Biotech, Shanghai, China) according to the manufacturer's instructions.

Quantitative real-time polymerase chain reaction

Quantitative RT-PCR was applied to examine the expression of EDA and dentin sialophosphoprotein (DSPP). Total RNA was reverse-transcribed using the Prime Script First Strand cDNA Synthesis Kit (TaKaRa Biotechnology, China), and RT-qPCR was carried out with SYBR Premix DimerEraser (TaKaRa) on a LightCycler 480 (Roche, Indianapolis, USA). The DSPP primers have been published elsewhere.

Alizarin Red S staining (ARS)

The number of calcium nodules formed by hDPSCs after transfection of EDA lentivirus vector, WT, or MUT EDA was analyzed by Alizarin Red S staining. After culture in the osteo/odontogenic medium (complete culture medium containing 50mg/mL ascorbic acid, 5mM b-glycerophosphate, and 10nM dexamethasone (Sigma-Aldrich)) for 14 days, the cells were fixed using 60% isopropanol and stained with 1% ARS (Sigma, USA) at room temperature.

Detecting Skewing of X Inactivation

The X-chromosome inactivation pattern was analyzed using the human androgen receptor (HUMARA) assay [24], which utilize the highly polymorphic CAG repeat in the first exon of the androgen receptor gene (AR). The AR gene has been used as a marker of skewed X chromosome inactivation through differential PCR amplification following digestion with the methylation-sensitive restriction enzyme HpaII. In brief, DNA samples were obtained from the peripheral venous blood of the female carrier (II3) and her affected father (I3). For each sample, 600ng of DNA was digested with HpaII. Digested products together with non-digested DNA were used as templates for amplification of the AR polymorphic repeat using fluorescence primers: AR, forward: 5'-GCTGTGAAGGTTGCTGTTCCTCAT-3' and reverse, 5'-

TCCAGAATCTGTTCCAGAGCGTGC-3’. And we identified the digestion efficiencies with MIC2 as internal reference. The amplification products of MIC2 can observed when the DNA was not complete digestion. The primers were as follows: MIC2F, forward: 5’-AGAGGTGCGTCCGATTTTTCCC-3’ and reverse, 5’-ACCGCCGCAGATGGACAATT-3’. The products were analyzed by capillary electrophoresis (Beckman Coulter, USA).

Bioinformatics

To further confirm the function of mutant EDA, the 3D structures of WT and mutant EDA were predicted in silico using I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER/), and the functional effects of the mutant protein were estimated with Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/).

Statistical analysis

Differences between two groups were analyzed using the Student’s t test using IBM SPSS Statistics 20 soft-ware (IBM Corp, NY, USA) and more than two groups were analyzed by one-way-ANOVA. $P < 0.05$ was considered significant.

Results

Clinical Phenotypes

Clinical and radiological examination revealed that the proband presented with 8 deciduous teeth missing and 18 permanent teeth missing (Fig.1b-c and Fig2b-b2). His brother (Fig.1b-c and Fig.2c-c2) had the same phenotypes with him, but the mother (Fig.1c and Fig.2a-a2) only had a tooth missed. The proband’s father and grandmother had no missing teeth. We only did blood collection from the grandfather without radiological examination because of his old age. And according to a description from other family members, the grandfather also had similar oral manifestations. All family members denied any medical history of abnormalities in other organs including the sweat glands, hair, skin, nails, or any other systemic disorder, indicating a NSH phenotype.

Mutation analysis

No mutations in PAX9, AXIN2, MSX1, or WNT10A, previously reported as candidate genes for isolated tooth agenesis, were detected by Sanger sequencing in this family. But we found a reported variant c.1013C > T (p. Thr338Met) in exon 8 of EDA. Sequencing analyses of the Thr338Met mutation was performed on all participants (Fig.3a). The affected female patient (II2) was heterozygous carrier for p. Thr338Met, and all affected male individuals (III1, III2, I3) were homozygous mutant, while unaffected grandmother (I4) did not carry this mutation (Fig.1 and Fig.3a).

Mutation of EDA inhibited osteo/odontogenic differentiation of hDPSCs

To investigate whether the mutant EDA affects the function of hDPSCs, hDPSCs were cultured with osteo/odontogenesis induction medium for 14 days after transfection of the Ctrl, WT and MUT, respectively, and calcium nodule deposition was examined by ARS staining. We found that EDA overexpression increased the expression of DSPP and the calcium nodule deposition after 14days of odontogenic induction, while the mutant EDA showed a decreased capacity for osteo/odontogenic differentiation compared with those transfected with WT (Fig.4a-c).

EDA regulates odontogenesis of hDPSCs through the NF-κB pathway

Western blot results showed that WT transfection significantly increased p65 and IκBα phosphorylation compared with the Ctrl，while mutant EDA transfection decreased p65 and IκBα phosphorylation in comparison with those transfected with wild-type EDA (Fig.4d). These results reveal, for the first time, that EDA regulates odontogenesis of hDPSCs via the NF-κB pathway，thus leading to the oligodontia phenotype in the patients.

Analysis of X Chromosome Inactivation Skewing

The results of capillary electrophoresis showed that there were three products of the II2 before DNA digestion: 284bp (paternal source), 293bp (maternal source) and 373bp (internal marker MIC2). And there was no product of MIC2 when the DNA was completely digested. Similarly, because men have only one X chromosome that cannot be methylated and thus there were two products before digestion and no products after digestion (Fig.5). In a word, the results indicated that the female carrier's X chromosome that obtained from paternal donor inactivation rate was 39.3%.

Discussion

We identified a known missense mutation (c.1013C>T, p.T338M) of EDA in a Chinese family which manifested as X-linked non-syndromic hypodontia [14]. Though previous studies have identified more than one hundred mutations related to XLHED in EDA, only a few EDA variants are reported as being implicated in non-syndromic tooth agenesis [25-27]. In addition, the EDA mutation site has been reported before, but the function of EDA in hDPSCs and whether the mutant EDA affects the function of hDPSCs haven’t been known. Human DPSCs are capable of both self-renewal and multilineage differentiation which play an essential part in dentin formation and regeneration [28-29]. Furthermore, normal differentiation of hDPSCs is essential for dentin development and formation. Therefore, hDPSCs represent a valuable model to investigate odontoblastic differentiation and to explore the mechanisms by which EDA affects the function of such cells.

Here, in the present study, we first confirmed intrinsic EDA expression and investigated its role in odontogenic differentiation of hDPSCs. To further analyze how the mutation of EDA caused tooth agenesis, we constructed wild-type EDA expression vectors and performed site-directed mutagenesis to obtain the mutant EDA expression vector, which were packaged into lentivirus to make hDPSCs easier to be infected. Wild-type EDA transfection increased calcium nodule formation and DSPP expression

compared with Ctrl. DPSCs transfected with mutant EDA lentivirus showed a decreased capacity for odontogenic differentiation with a lower expression level of DSPP compared with those transfected with wild-type EDA lentivirus. To our knowledge, this study is the first to demonstrate the effects of EDA and its mutant on hDPSCs.

Previous studies have confirmed the NF-κB pathway as a downstream effector of EDA [8]. The EDA/NF-κB pathway has been found to be important for tooth growth, and mutations in several genes in this pathway have been reported to be responsible for ectodermal dysplasia, such as syndromic tooth agenesis [30-31]. Therefore, dysfunctional EDA might severely impair the NF-κB activations in various stages during tooth development, thus leading to abnormalities in the permanent dentition and even in the primary dentition. However, whether EDA and the mutant EDA affect NF-κB pathway of hDPSCs are yet to be elucidated. In our studies, we confirmed the relationship between the EDA mutation and NF-κB signaling pathway in hDPSCs. But why the EDA mutation can partially, not completely abolish the activation of NF-κB need to be further investigated.

Previous studies about EDA mutation suggested that the male patients' clinical symptoms and phenotype were more severe than the female carriers [15-16]. Skewed inactivation of the X-chromosome, with over-expression of the mutant gene in affected females, could theoretically be the cause of oligodontia phenotype [18-20]. In this family, the female carrier (II2) only had one tooth missed that was different from the previously reported case (several missing teeth) of the same mutation site, while her sons with mutation had a severe phenotype. In order to verify whether skewed X-inactivation may be involved in the pathogenesis of disease in female carrier in this family, we conducted XCI analysis in the peripheral blood of the female carrier. Even though the results shown only a 39.3:61.7 skewing of X inactivation in the peripheral blood DNA as measured by androgen receptor allele methylation, we can still partly explain why the female carrier has a milder phenotype. We can understand like this, if the pattern of inheritance in our pedigree is X-linked recessive and at the same time, the X chromosome inactivation rate is 50:50, the female carrier will not have tooth agenesis. If, however, there is a slightly skewed inactivation of the X-chromosome, the female carrier will have a mild phenotypes of tooth agenesis. Similarly，if there is a severely skewed inactivation of the X-chromosome (≥80:20), the female carrier will have a severe phenotypes of tooth agenesis.

Conclusion

In summary, we verified the pathogenicity of the EDA c.1013C>T mutation in a three-generation Chinese family with X-linked non-syndromic hypodontia. Subsequent in-vitro studies, for the first time, verified the odontogenic function of EDA and its mutant in hDPSCs. And the female mild phenotype might involve X-chromosome inactivation. Understanding possible mechanisms will provide insights into novel therapeutic strategies for patients with tooth agenesis caused by EDA mutation in the future.

Abbreviations

EDA: Ectodysplasin-A; hDPSCs: human dental pulp stem cells; CE: capillary electrophoresis; XCI: X chromosome inactivation; DSPP: dentin sialophosphoprotein; NSH: non-syndromic hypodontia; SH: syndromic hypodontia; XLHED: X-linked hypohidrotic ectodermal dysplasia; PCR: Polymerase chain reaction; WT: Wild type; MUT: Mutant type; OM: Odontogenic medium; DMEM: Dulbecco's modified Eagle's medium; FBS: Fetal bovine serum; MOI: Multiplicity of infection; ARS：Alizarin Red S staining; HUMARA: human androgen receptor; AR: androgen receptor; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; PAX9: Paired box 9; WNT10A: Wnt family member 10A; MSX1: Msh homeobox 1; AXIN2: Axis inhibition protein 2; NF-κB: Nuclear factor kappa-B; MIC2: Micronemal protein 2; Thr: Threonine; Met: Methionine. EDAR: Ectodysplasin-A receptor; EDARADD: EDAR associated death domain; IKBKG: Inhibitor of nuclear factor kappa B kinase regulatory subunit gamma.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

YHP, TL and LP, contributed to design, data acquisition, and analysis, drafted and critically revised the manuscript; XYH and XCY contributed to data acquisition and analysis, critically revised the manuscript; BLW and FX, contributed to conception, design, data analysis, and interpretation, critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

Funding

This work was supported by the National Natural Science Foundation of China (31970558, 81870755) and Natural Science Foundation of Guangdong Province of China (2018B030311033).

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Nanfang Hospital, Southern Medical University. Written informed consent was obtained from all adult patients.

Consent for publication

Not applicable.

Competing interests

The authors have declared no competing interests.

References

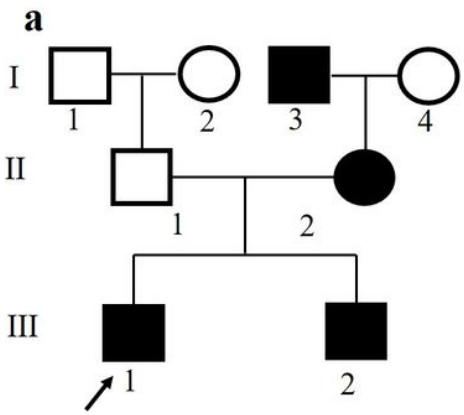
1. Choi SJ, Lee JW, Song JH. Dental anomaly patterns associated with tooth agenesis. Acta Odontol Scand. 2017;75(3):161-165.
2. Al-Ani AH, Antoun JS, Thomson WM, Merriman TR, Farella M. Hypodontia: An Update on Its Etiology, Classification, and Clinical Management. Biomed Res Int. 2017;2017:9378325.
3. Matalova E, Fleischmannova J, Sharpe PT, Tucker AS. Tooth agenesis: from molecular genetics to molecular dentistry. J Dent Res. 2008;87(7):617-23.
4. AlNuaimi R, Mansoor M. Prosthetic rehabilitation with fixed prosthesis of a 5-year-old child with Hypohidrotic Ectodermal Dysplasia and Oligodontia: a case report. J Med Case Rep. 2019;13(1):329.
5. Pirgon O, Atabek ME, Tanju IA. Congenital anodontia in ectodermal dysplasia. J Pediatr Endocrinol Metab. 2008;21(12):1111-2.
6. Mártha K, Kerekes Máthé B, Moldovan VG, Bănescu C. Study of rs12532, rs8670 Polymorphism of Msh Homeobox 1 (MSX1), rs61754301, rs4904155 Polymorphism of Paired Box Gene 9 (PAX9), and rs2240308 Polymorphism of Axis Inhibitor Protein 2 (AXIN2) Genes in Nonsyndromic Hypodontia. Biomed Res Int. 2019;2019:2183720.
7. Magruder S, Carter E, Williams MA, English J, Akyalcin S, Letra A. Further evidence for the role of WNT10A, WNT10B and GREM2 as candidate genes for isolated tooth agenesis. Orthod Craniofac Res. 2018;21(4):258-263.
8. Kowalczyk-Quintas C, Schneider P. Ectodysplasin A (EDA)-EDA receptor signalling and its pharmacological modulation. Cytokine Growth Factor Rev. 2014;25(2):195-203.
9. Masui Y, Farooq M, Sato N, Fujimoto A, Fujikawa H, Ito M, Shimomura Y. A missense mutation in the death domain of EDAR abolishes the interaction with EDARADD and underlies hypohidrotic ectodermal dysplasia. Dermatology. 2011;223(1):74-9.
10. Yan M, Wang LC, Hymowitz SG, Schilbach S, Lee J, Goddard A, de Vos AM, Gao WQ, Dixit VM. Two-amino acid molecular switch in an epithelial morphogen that regulates binding to two distinct receptors. Science. 2000;290(5491):523-7.
11. Yu M, Wong SW, Han D, Cai T. Genetic analysis: Wnt and other pathways in nonsyndromic tooth agenesis. Oral Dis. 2019;25(3):646-651.
12. Zhao K, Lian M, Zou D, Huang W, Zhou W, Shen Y, Wang F, Wu Y. Novel mutations identified in patients with tooth agenesis by whole-exome sequencing. Oral Dis. 2019;25(2):523-534.
13. Mues G, Tardivel A, Willen L, Kapadia H, Seaman R, Frazier-Bowers S, Schneider P, D'Souza RN. Functional analysis of Ectodysplasin-A mutations causing selective tooth agenesis. Eur J Hum Genet. 2010;18(1):19-25.
14. Han D, Gong Y, Wu H, Zhang X, Yan M, Wang X, Qu H, Feng H, Song S. Novel EDA mutation resulting in X-linked non-syndromic hypodontia and the pattern of EDA-associated isolated tooth agenesis. Eur J Med Genet. 2008;51(6):536-46.

15. Khabour OF, Mesmar FS, Al-Tamimi F, Al-Batayneh OB, Owais AI. Missense mutation of the EDA gene in a Jordanian family with X-linked hypohidrotic ectodermal dysplasia: phenotypic appearance and speech problems. Genet Mol Res. 2010;9(2):941-8.
16. Zhang L, Yu M, Wong SW, Qu H, Cai T, Liu Y, Liu H, Fan Z, Zheng J, Zhou Y, Feng H, Han D. Comparative analysis of rare EDAR mutations and tooth agenesis pattern in EDAR- and EDA-associated nonsyndromic oligodontia. Hum Mutat. 2020;41(11):1957-1966.
17. Lexner MO, Bardow A, Juncker I, Jensen LG, Almer L, Kreiborg S, Hertz JM. X-linked hypohidrotic ectodermal dysplasia. Genetic and dental findings in 67 Danish patients from 19 families. Clin Genet. 2008;74(3):252-9.
18. Wang J, Yu R, Shete S. X-chromosome genetic association test accounting for X-inactivation, skewed X-inactivation, and escape from X-inactivation. Genet Epidemiol. 2014;38(6):483-93.
19. Disteche CM, Berletch JB. X-chromosome inactivation and escape. J Genet. 2015;94(4):591-9.
20. Schurz H, Salie M, Tromp G, Hoal EG, Kinnear CJ, Möller M. The X chromosome and sex-specific effects in infectious disease susceptibility. Hum Genomics. 2019;13(1):2.
21. Viggiano E, Picillo E, Ergoli M, Cirillo A, Del Gaudio S, Politano L. Skewed X-chromosome inactivation plays a crucial role in the onset of symptoms in carriers of Becker muscular dystrophy. J Gene Med. 2017;19(4).
22. Wu C, Sun Z. X chromosome abnormal inactivation: a unique factor for women's diseases? Epigenomics. 2016;8(4):447-50.
23. Pan Y, Lu T, Peng L, Chen Z, Li M, Zhang K, Xiong F, Wu B. Vacuolar protein sorting 4B regulates the proliferation and odontoblastic differentiation of human dental pulp stem cells through the Wnt-β-catenin signalling pathway. Artif Cells Nanomed Biotechnol. 2019;47(1):2575-2584.
24. Allen RC, Zoghbi HY, Moseley AB, Rosenblatt HM, Belmont JW. Methylation of HpaII and HhaI sites near the polymorphic CAG repeat in the human androgen-receptor gene correlates with X chromosome inactivation. Am J Hum Genet. 1992;51(6):1229-39.
25. Andreoni F, Sgattoni C, Bencardino D, Simonetti O, Forabosco A, Magnani M. Missense mutations in EDA and EDAR genes cause dominant syndromic tooth agenesis. Mol Genet Genomic Med. 2020;18:e1555.
26. Zeng B, Zhao Q, Li S, Lu H, Lu J, Ma L, Zhao W, Yu D. Novel EDA or EDAR Mutations Identified in Patients with X-Linked Hypohidrotic Ectodermal Dysplasia or Non-Syndromic Tooth Agenesis. Genes (Basel). 2017;8(10):259.
27. Huang SX, Liang JL, Sui WG, Lin H, Xue W, Chen JJ, Zhang Y, Gong WW, Dai Y, Ou ML. EDA mutation as a cause of hypohidrotic ectodermal dysplasia: a case report and review of the literature. Genet Mol Res. 2015;14(3):10344-51.
28. Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proc Natl Acad Sci U S A. 2000;97(25):13625-30.
29. Daltoé FP, Mendonça PP, Mantesso A, Deboni MC. Can SHED or DPSCs be used to repair/regenerate non-dental tissues? A systematic review of in vivo studies. Braz Oral Res. 2014;28:S1806-

83242014000100401.

30. Sun S, Li F, Liu Y, Qu H, Wong SW, Zeng L, Yu M, Feng H, Liu H, Han D. A novel inhibitor of nuclear factor kappa-B kinase subunit gamma mutation identified in an incontinentia pigmenti patient with syndromic tooth agenesis. Arch Oral Biol. 2019;101:100-107.
31. Zonana J, Elder ME, Schneider LC, Orlow SJ, Moss C, Golabi M, Shapira SK, Farndon PA, Wara DW, Emmal SA, Ferguson BM. A novel X-linked disorder of immune deficiency and hypohidrotic ectodermal dysplasia is allelic to incontinentia pigmenti and due to mutations in IKK-gamma (NEMO). Am J Hum Genet. 2000;67(6):1555-62.

Figures



b

Patient	MT		Right quadrants					Left quadrants				
			5	4	3	2	1	1	2	3	4	5
III:1	8	Maxillary		*		*			*		*	
		Mandibular				*	*	*	*			
III:2	8	Maxillary		*		*			*		*	
		Mandibular				*	*	*	*			

c

Patient	MT		Right quadrants								Left quadrants							
			8	7	6	5	4	3	2	1	1	2	3	4	5	6	7	8
II:2	1	Maxillary									*							
		Mandibular																
III:1	18	Maxillary	*			*	*		*			*		*	*			*
		Mandibular	*			*	*		*	*	*	*		*	*			*
III:2	18	Maxillary	*			*	*		*			*		*	*			
		Mandibular	*			*	*		*	*	*	*		*	*			*

Figure 1

a Pedigree of the family. Males are marked as squares and females as circles. An arrow indicates the proband, and the black symbols indicate the affected individuals. b Summary of missing primary teeth of the two male patients whose panoramic radiographs. c Summary of missing permanent teeth of the three patients whose panoramic radiographs.

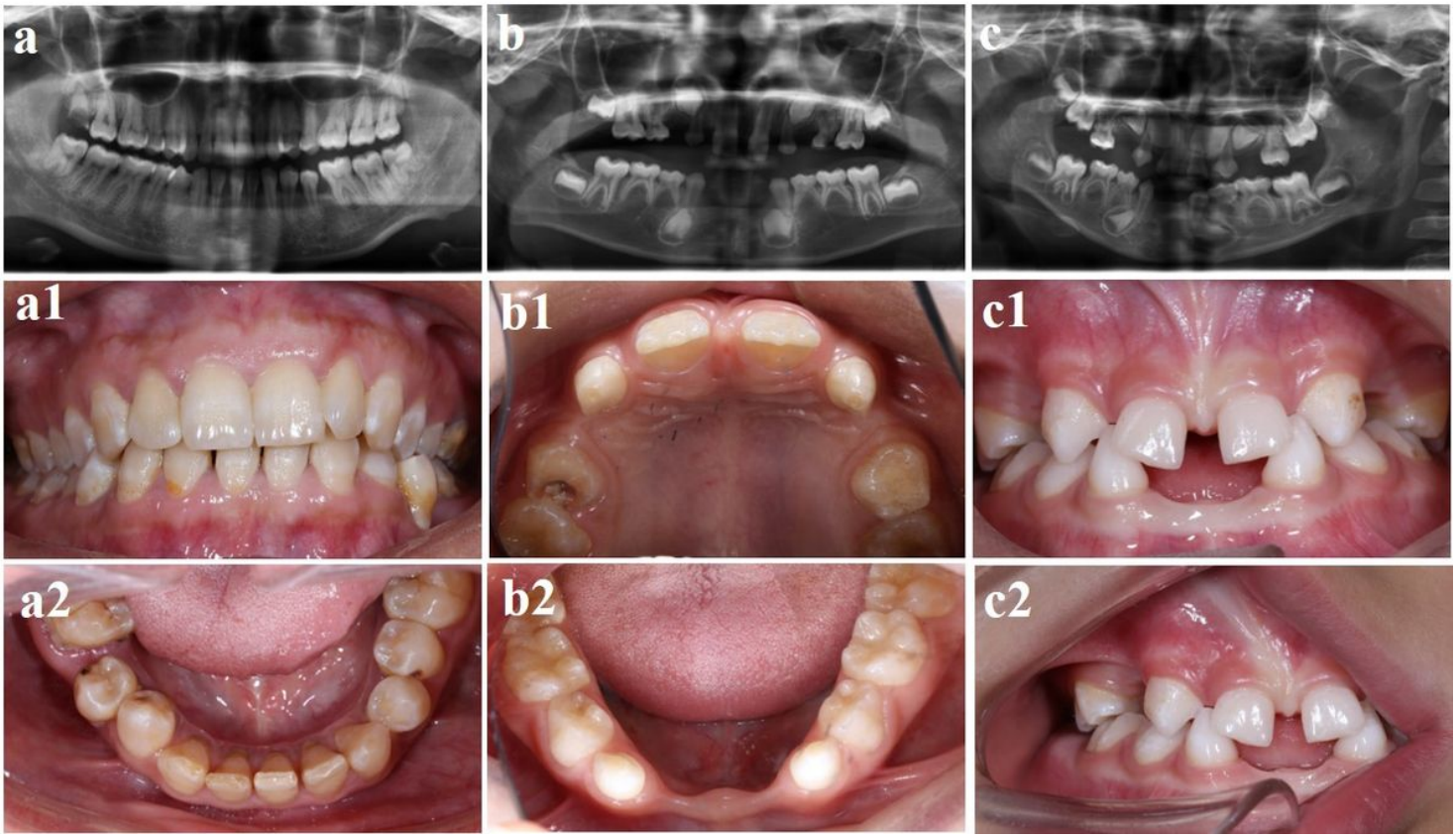


Figure 2

Dental phenotype and panoramic radiograph of the patients. a-c Panoramic radiograph of the II:2, III:1 and III:2 respectively. a1-a2, b1-b2 and c1-c2 Intraoral photos of the II:2, III:1 and III:2 respectively.

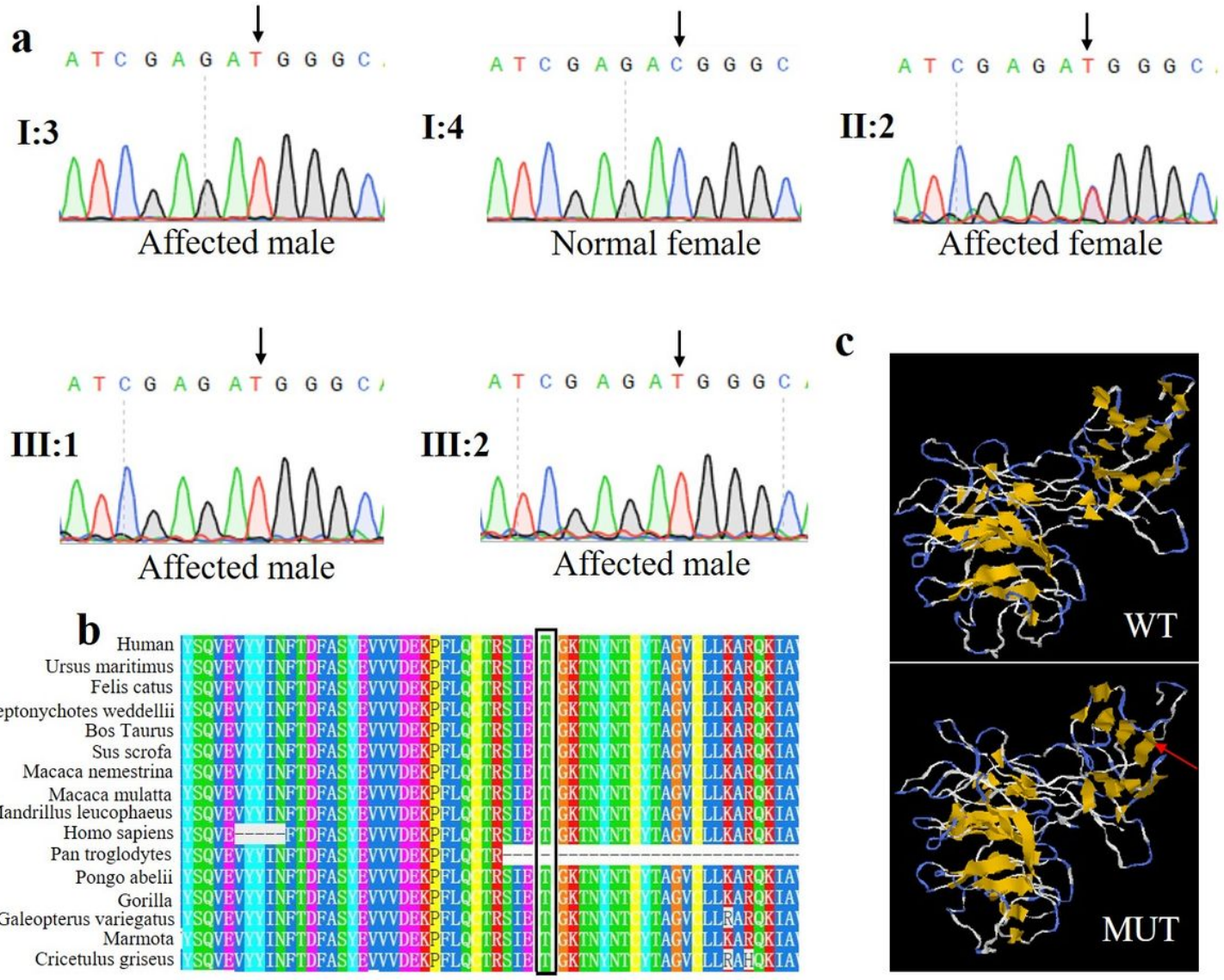


Figure 3

Mutation analysis. a Mutation screening. Sanger sequencing results of the wild-type and the mutant. b Conservation of the mutant EDA across species. c Three-dimensional models of wild-type and mutant EDA.

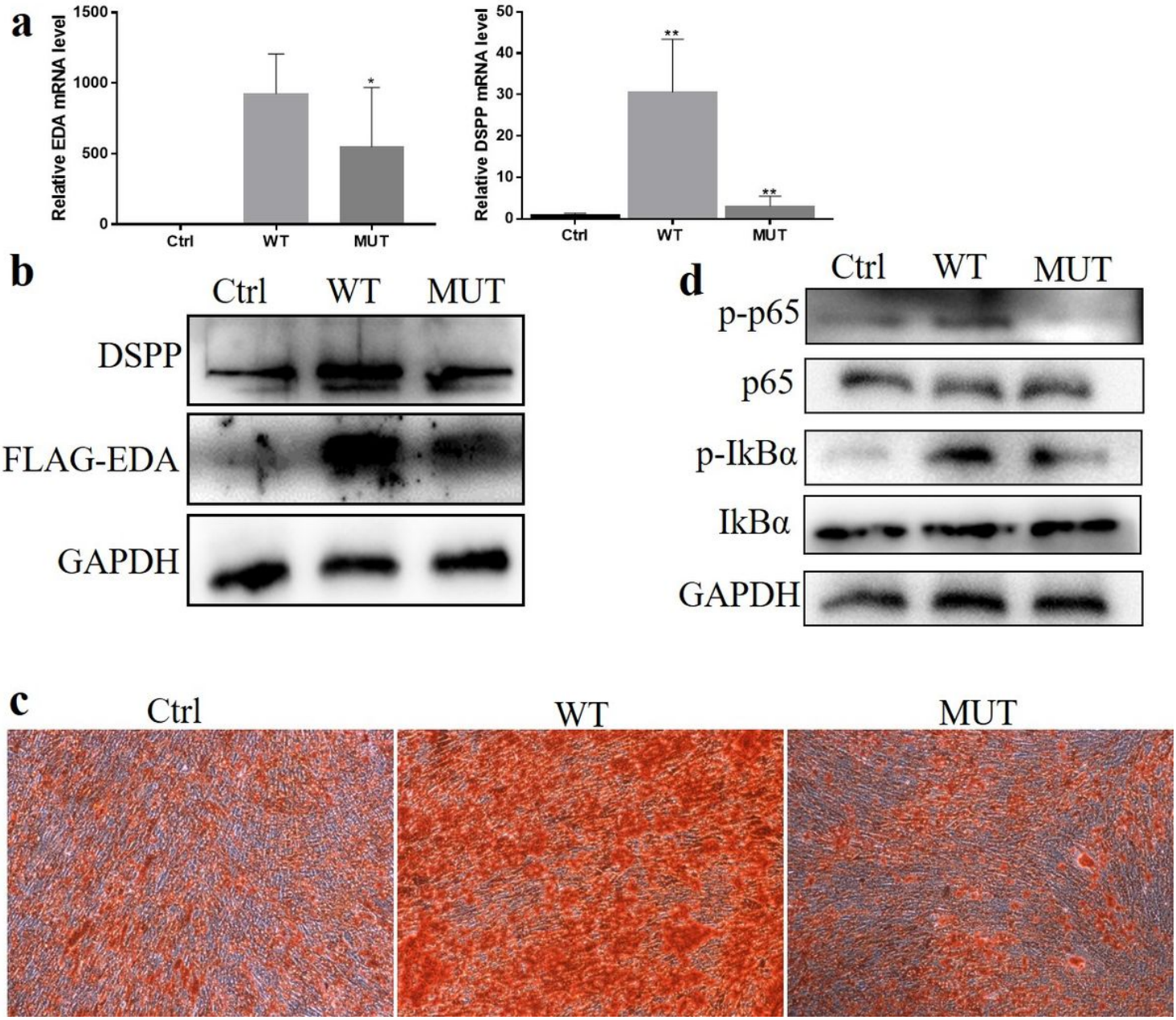


Figure 4

Effects of EDA on odontogenic differentiation of DPSCs. a mRNA expression level of EDA and DSPP in hDPSCs. There was a significant difference in the mRNA level of the wild-type and the mutant. b Western blot analysis of EDA and DSPP expression. c Calcium nodule deposition of DPSCs after transfection was examined by Alizarin Red S staining. Mutant (MUT) EDA showed decreased capacity of odontogenic differentiation compared with those transfected with wild-type (WT) EDA. d DPSCs transfected with mutant EDA expressed lower levels of p-p65 and p-IkBαin comparison with wild-type EDA. Data are expressed as the mean ± SD. Each experiment was repeated three times with n ≥3 samples per group. *P< 0.05, **P< 0.01.

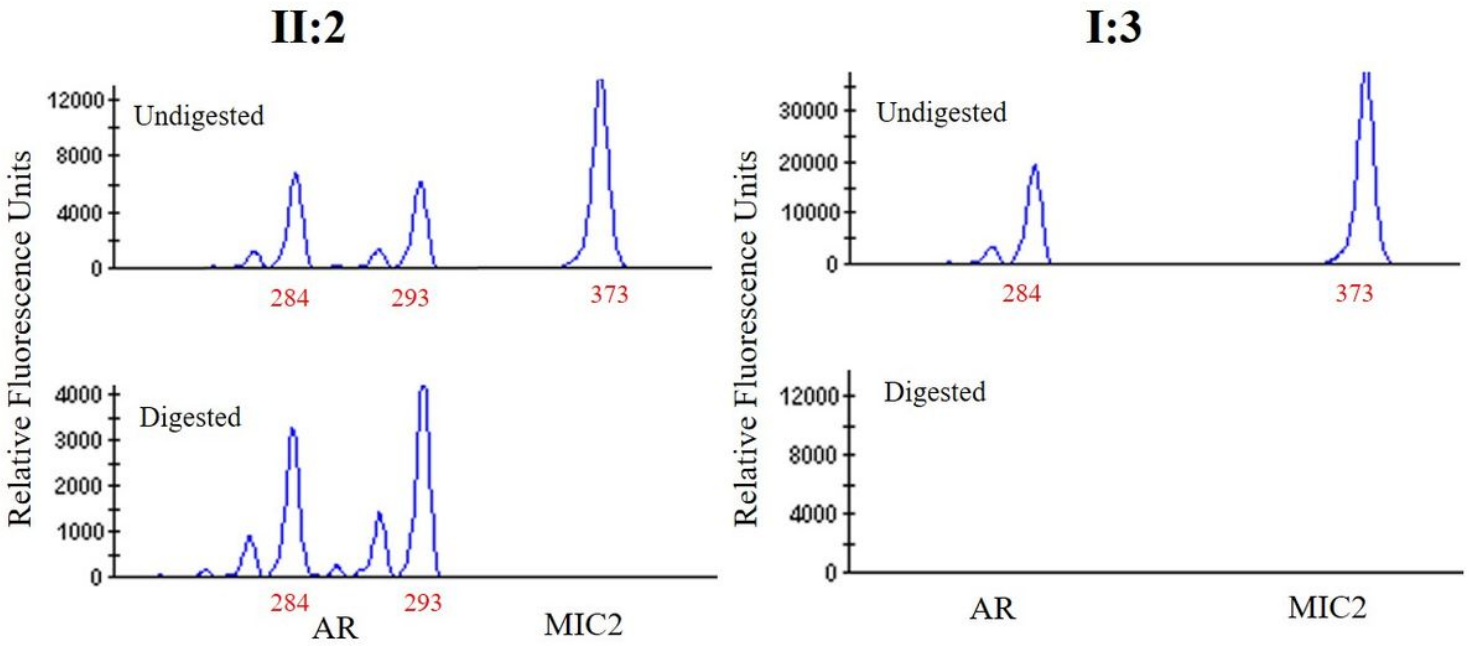


Figure 5

Skewing of X inactivation analysis. The HUMARA assay was performed on genomic DNA extracted from the heterozygous female patient (II:2) and her father's (I:3) peripheral blood. The DNA was digested with and without a methylation-sensitive restriction enzyme (HpaII), amplified by PCR using FAM-labeled primers specific to the HUMARA locus, and analyzed by capillary electrophoresis.