REST Final-Exon-Truncating Mutations Cause Hereditary Gingival Fibromatosis

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Hereditary gingival fibromatosis (HGF) is the most common genetic form of gingival fibromatosis that develops as a slowly progressive, benign, localized or generalized enlargement of keratinized gingiva. HGF is a genetically heterogeneous disorder and can be transmitted either as an autosomal-dominant or autosomal-recessive trait or appear sporadically. To date, four loci (2p22.1, 2p23.3–p22.3, 5q13–q22, and 11p15) have been mapped to autosomes and one gene (*SOS1*) has been associated with the HGF trait observed to segregate in a dominant inheritance pattern. Here we report 11 individuals with HGF from three unrelated families. Whole-exome sequencing (WES) revealed three different truncating mutations including two frameshifts and one nonsense variant in RE1-silencing transcription factor (*REST*) in the probands from all families and further genetic and genomic analyses confirmed the WES-identified findings. REST is a transcriptional repressor that is expressed throughout the body; it has different roles in different cellular contexts, such as oncogenic and tumor-suppressor functions and hematopoietic and cardiac differentiation. Here we show the consequences of germline final-exon-truncating mutations in *REST* for organismal development and the association with the HGF phenotype.

Gingival fibromatosis (GF) is a rare condition of gingival overgrowth characterized by a slowly progressive, benign, localized or generalized fibrous enlargement of maxillary and mandibular keratinized gingiva. GF may co-exist with various genetic syndromes (i.e., generalized hypertrichosis terminalis [MIM: 135400], Zimmermann-Laband syndrome [MIM: 135500], amelogenesis imperfecta type IG [MIM: 204690], hyaline fibromatosis syndrome [MIM: 228600], etc.) or occurs as an apparent isolated trait as non-syndromic hereditary gingival fibromatosis (HGF).[1] GF may also develop from environmental exposure such as a side effect of medications including anticonvulsants (i.e., phenytoin), immunosuppressants (i.e., cyclosporine), or calcium channel blockers (i.e., nifedipine, diltiazem, and verapamil).[2,3] The initial differential diagnosis for GF also includes chronic hyperplastic gingivitis, leukemic infiltrate, and some systemic diseases such as Crohn disease (MIM: 266600), neurofibromatosis (MIM: 162200), primary amyloidosis (MIM: 204850), sarcoidosis (MIM: 181000), scurvy (MIM: 240400), and Wegener granulomatosis (MIM: 608710) that have been associated with gingival overgrowth.[4]

HGF is the most common genetic form of GF which is usually transmitted as an autosomal-dominant trait. Simplex cases are also common and autosomal-recessive inheritance has been reported.[5,6] To date, four loci (2p22.1 [MIM: 135300], 5q13–q22 [MIM: 605544], 2p23.3–p22.3 [MIM: 609955], and 11p15 [MIM: 611010]) have been associated with HGF; a heterozygous frameshift mutation in *SOS1* (MIM: 182530) has been reported as the cause of autosomal-dominant HGF in a family showing linkage to 2p21.[7] The estimated frequency of HGF is 1:175,000, equally affecting males and females.[1] The surgical treatment, gingivectomy with gingivoplasty, can be applied to individuals with massive gingival enlargement in cases of aesthetic concern; nevertheless, the recurrence of hyperplasia is relatively high potentially because of the genetic predisposition especially in hereditary forms.

We report three unrelated families with eleven affected individuals clinically diagnosed with GF. Genomic analysis of these families led to the identification of three different heterozygous mutations predicted by conceptual translation to result in premature termination of the nascent transcript, two frameshifts, and one nonsense allele, in the RE1-silencing transcription factor gene (*REST*).

Two affected siblings (II-1 and II-2 in Figure 1A) and their mildly affected father (I-1 in Figure 1A) from family 1 were referred to Marmara University School of Medicine, and six affected individuals (II-2, II-5, II-6, III-1, III-3, and III-5 in Figure 1B) from family 2 were referred to Istanbul Faculty

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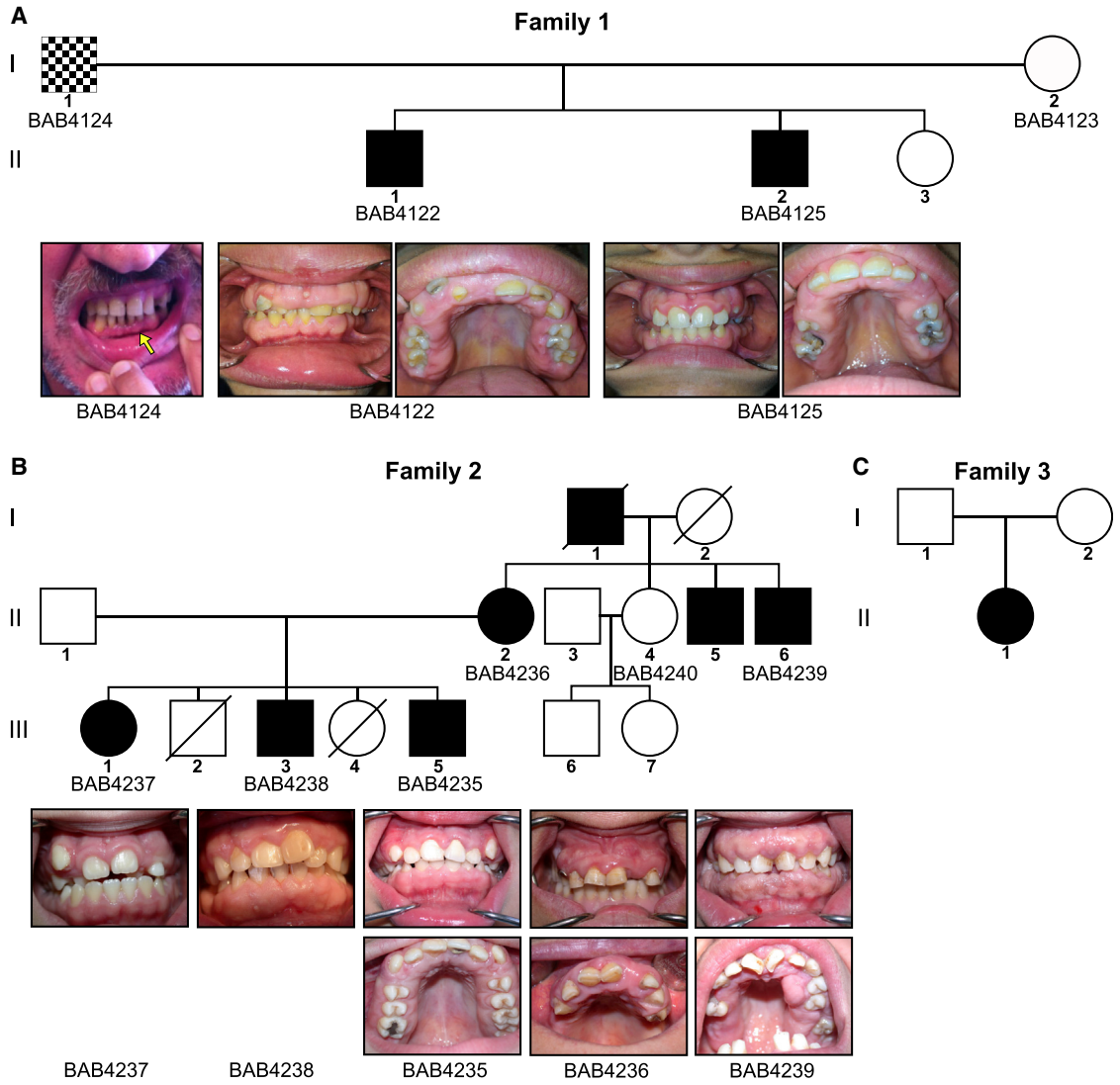

Figure 1. Pedigrees and Gingival Photographs of the Individuals Studied
Black-filled boxes indicate affected individuals in families 1 (A), 2 (B), and 3 (C) and checkerboard-filled box indicates mildly affected father in family 1. Visual inspection reveals the gingival overgrowth. Note the mild GF prominently involving the mandibular arch (yellow arrow) in the father (BAB4124, I-1) in family 1.

of Medicine, in Turkey. Intraoral examination revealed severe generalized GF in all affected individuals in both families except the father (BAB4124, I-1 in Figure 1A) in family 1. His clinical examination revealed mild gingival overgrowth while both of his affected sons (BAB4122, II-1 and BAB4125, II-2 in Figure 1A) had more severe GF. The deceased maternal grandfather in family 2 (I-1 in Figure 1B) was also reported to have similar signs of gingival enlargement. Exposure to any medication that may cause GF was excluded in all individuals. Genetic analysis including karyotype and *SOS1* mutation screening on the proband in family 2 prior to whole-exome sequencing (WES) revealed normal results. The clinical findings of affected individuals with available data are summarized in Table 1; detailed case reports can be found in the Supplemental Note and photos are available in Figure 1. Additionally, a case report of family 2 including clinical findings, karyotype, and *SOS1* mutation analysis results was previously published by Pehlivan et al.[8]

After informed consent was obtained from all participants according to the Baylor-Hopkins Center for Mendelian Genomics (BHCMG) research protocol (IRB protocol number: H-29697), WES was performed on probands (BAB4122, II-1 in Figure 1A and BAB4235, III-5 in Figure 1B) in families 1 and 2 according to previously described protocols.[9] In brief, genomic DNA samples underwent whole-exome capture using VCRome 2.1 design[10] (42 Mb NimbleGen, Cat. No. 06266380001) according to the manufacturer's protocol (NimbleGen SeqCap EZ Exome Library SR User's Guide) with minor modifications, followed by sequencing on the HiSeq platform (Illumina) with a sequencing yield of 8.4 Gb; the samples achieved 96% of the targeted exome bases covered to a depth-of-coverage of 20× or greater.[11] Data produced were aligned and

Table 1. Clinical Summary of the Individuals with *REST* Variants

	Family 1			Family 2					Family 3
	BAB4122	**BAB4124**	**BAB4125**	**BAB4235**	**BAB4236**	**BAB4237**	**BAB4238**	**BAB4239**	**Proband**
Identified variant	p.Asn958Serfs*9	p.Asn958Serfs*9 (mosaic)	p.Asn958Serfs*9	p.Leu437*	p.Leu437*	p.Leu437*	p.Leu437*	p.Leu437*	p.Leu805Phefs*38
Age at last exam	17 years	48 years	13 years	9 years	38 years	14 years	16 years	32 years	9 years
Gender	male	male	male	male	female	female	male	male	female
Gingival fibromatosis	+	+ (mild)	+	+	+	+	+	+	+
Age of onset	7–8 years	NA	7–8 years	3–4 years	3–4 years	3–4 years	3–4 years	3–4 years	3–4 years
Exposure to medication	no	no	no	no	no	no	no	no	no
Gingivectomy/ recurrence	+/NA	NA/NA	+/NA	NA/NA	NA/NA	NA/NA	NA/NA	NA/NA	+/+
Pectus deformity	+	no	+	no	no	no	no	no	+
Other clinical findings	osteomyelitis, osteoporosis	NA	NA	NA	NA	NA	NA	NA	small hiatal hernia, short stature, recurrent URIs

Abbreviations are as follows: NA, not available; URIs, upper respiratory infections

mapped to the human genome reference sequence (hg19) using the Mercury in-house bioinformatics pipeline.[12] Variants were called using the ATLAS variant calling method and the Sequence Alignment/Map (SAMtools) suites and annotated with an in-house-developed Cassandra annotation pipeline that uses Annotation of Genetic Variants (ANNOVAR) and additional tools and databases. Then, we applied variant prioritization for indels as the following. If an indel is reported as a variant in the Human Gene Mutation Database (HGMD) and its frequency is less than 5% in the 1000 Genomes Project (1000GP) data, it was included. If it is not present in HGMD, it has to pass through all of the variant quality filters and its frequency has to be less than 2% in 1000GP data to be prioritized and investigated further as a potential pathogenic variant. In a second filtering step, another filter is further applied based on the number of samples having the variant in Atherosclerosis Risk in Communities Study (ARIC) database. The number of samples having the variant in the ARIC database should be less than 120 out of 10,940 samples in total. For single nucleotide variants (SNVs), we applied different filtering criteria. If a SNV is reported as a variant in the HGMD or it has a clinical variant value between 3 and 8 in the Single Nucleotide Polymorphism database (dbSNP), its frequency has to be less than 5% in both 1000GP data and NHLBI GO Exome Sequencing Project (ESP5400) African and European populations. If it is not present in HGMD and does not have a clinical variant value between 3 and 8 in dbSNP, it has to pass through all of the variant quality filters and its frequency has to be less than 1% in both 1000GP data and ESP5400 African and European populations to be prioritized and investigated further as a potential pathogenic variant. Like indels, the SNVs that pass these filters were further filtered based on the number of samples having the variant in the ARIC database (should be less than 120 out of 10,940 samples in total). At the end of those filtering steps for SNVs and indels, we obtained ~800 variants on average for each sample.

Given the apparent autosomal-dominant inheritance pattern in the pedigrees of families 1 and 2, we focused on the heterozygous variants in the parsed and filtered WES data. Out of ~800 variants, we selected the heterozygous variants that have an allele frequency below 0.1% in our internal database (CMG), which consists of more than 6,500 exomes including ~1,050 Turkish exomes. Then potential pathogenic variants including indels, nonsense and splice site variants, and missense variants that were predicted as deleterious in at least three out of five computational algorithms including SIFT, PolyPhen-2, LRT, MutationTaster, and PROVEAN were selected as candidate variants. After these filtering steps, ~50 heterozygous candidate variants remained for manual inspection from the personal genome analysis for each individual. The comparative analysis of the filtered WES variants revealed that deleterious mutations in *REST* were shared in the unrelated individuals. We identified two different heterozygous truncating variants in *REST* including a frameshift deletion in subject BAB4122 (family 1, II-1 in Figure 1A) (GenBank: NM_005612.4; exon 4; c.2865_2866delAA [p.Asn958Serfs*9]; chr4:57,797,888_CAA>C; variant to total reads = 5/19) and a nonsense variant in subject BAB4235 (family 2, III-5 in Figure 1B) (GenBank: NM_005612.4; exon 4; c.1310T>A [p.Leu437*]; chr4:57,796,334_T>A; variant to total reads = 18/33).

Additionally, after those above-mentioned filtering steps, we extracted all frameshift and stopgain variants in our CMG database and developed a computational workflow to predict whether they are likely to be subject to nonsense-mediated decay (NMD-competent) or escape from nonsense-mediated decay (NMD-incompetent) based on the location of the conceptually translated premature termination codon (50 bp rule). Then for each gene, we counted the number of NMD-competent and NMD-incompetent variants and identified the genes that are enriched for NMD-incompetent variants compared to NMD-competent variants (unpublished results). *REST* represented one of the genes identified by this computational approach, having two distinct NMD-incompetent variants (the variants that were identified in both individuals with GF), but no NMD-competent variants in the CMG database.

After we identified these extremely rare variants by two independent computational filtering analyses methods, we submitted *REST* to GeneMatcher, which is an online matching tool for connecting researchers with an interest in the same gene.[13] The GeneMatcher tool facilitated identification of family 3, which has an affected child (II-1 in Figure 1C) with GF. The female proband was initially evaluated at the Division of Medical Genetics Children's Hospital of Orange County in California at 9 years of age due to gingival overgrowth. Other remarkable clinical findings of the affected individual were small hiatal hernia, pectus excavatum, short stature, and recurrent upper respiratory infections. Family 3 was sequenced by a trio approach and a de novo frameshift *REST* variant (GenBank: NM_005612.4; exon 4; c.2413delC [p.Leu805Phefs*38]; chr4:57,797,436_CC>C; variant to total reads = 36/71) was identified (see Supplemental Note for detailed downstream WES). None of these three truncating alleles have been reported before in our CMG database or in other publicly available databases such as the Exome Aggregation Consortium (ExAC) database.

To confirm and evaluate potential disease-associated variants for co-segregation with the phenotype in all family members, with available DNA, we performed conventional PCR. Exon four of *REST* was amplified from genomic DNA by using the primer sets provided in the Supplemental Note. The resulting PCR products were purified with ExoSAP-IT (Affymetrix) and sequenced using Sanger di-deoxy nucleotide sequencing.

In family 1 both affected siblings (BAB4122, II-1 and BAB4125, II-2 in Figure 1A) were found to be heterozygous for the identified frameshift c.2865_2866delAA (p.Asn958Serfs*9) variant allele and the mother was found to be wild-type (Figure 2A). The mildly affected father also appeared to contain two wild-type alleles by Sanger sequencing; however, the observed mild GF phenotype in the father suggested a possible tissue-specific mosaicism and transmission of the frameshift variant to the affected siblings from the father. To confirm the putative potential mosaicism, we collected a buccal smear sample from the father and subjected the isolated DNA to Sanger sequencing. The Sanger chromatogram result revealed evidence for subtle secondary peaks that begin at the position in the sequence of the frameshift mutation observed in the two affected children (Figure 2A). This observation in the buccal DNA sample from the father, in combination with the observation of both affected siblings sharing the same heterozygous rare variant allele, provides evidence suggesting mosaicism in the father. Additionally, we inspected all resultant variants in the BAM file of the exome-sequenced index case (BAB4122, II-1 in Figure 1A) with the Integrative Genomics Viewer[14] and we found a heterozygous SNP (rs3796529) nearby the frameshift variant; subsequent Sanger sequencing of blood DNA from all family members revealed that this SNP is informative as the father is homozygous, the affected brothers were heterozygous, and the mother was wild-type for the identified SNP (Figure S1). Moreover, we manually cloned the entire region encompassing the SNP and frameshift allele in the affected brothers; individual clonal colonies were sequenced by a standard Sanger capillary protocol. Using this method, we observed that the frameshift is in *cis* with the SNP in the affected brothers (Figure S1). This indicates that the father's haplotype segregates with the mutant allele, providing further evidence that the mild GF present in the father is probably due to low-level mosaicism. In a further experiment to investigate mosaicism in the father, we performed restriction enzyme analysis on DNA from buccal and blood samples of the father and blood samples from the affected brothers and mother. This experimental approach yielded two bands in both affected brothers but not in the unaffected parents, including the father with suspected mosaicism (Figure S2).

In family 2, Sanger sequencing revealed that all five available affected individuals, including the proband, two siblings, mother, and a maternal uncle (III-5, III-1, III-3, II-2, and II-6, respectively, in Figure 1B), were heterozygous carriers for the WES-identified stop-gain variant (c.1310T>A [p.Leu437*]) and the unaffected maternal aunt (II-4 in Figure 1B) was wild-type at this locus (Figure 2A). In family 3 the de novo *REST* variant (c.2413delC [p.Leu805fsPhe*38]) was identified in the proband (II-1 in Figure 1C) by a trio WES approach; it was also confirmed by Sanger sequencing (Figure 2A).

From a clinical perspective, the proband in family 1 (BAB4122, II-1 in Figure 1A) had a more severe phenotype with the clinical findings of osteoporosis and osteomyelitis compared to his affected brother, and the proband (II-1 in Figure 1C) from family 3 had some additional clinical findings such as short stature first detected at age 12, hiatal hernia, and recurrent upper respiratory infections. Additionally, both brothers in family 1 (II-1 and II-2 in Figure 1A) as well as the proband in family 3 (II-1 in Figure 1C) had a pectus deformity. However, no other pathogenic variants that may potentially explain the additional findings observed in these individuals were identified during further examination of parsed and filtered WES data.

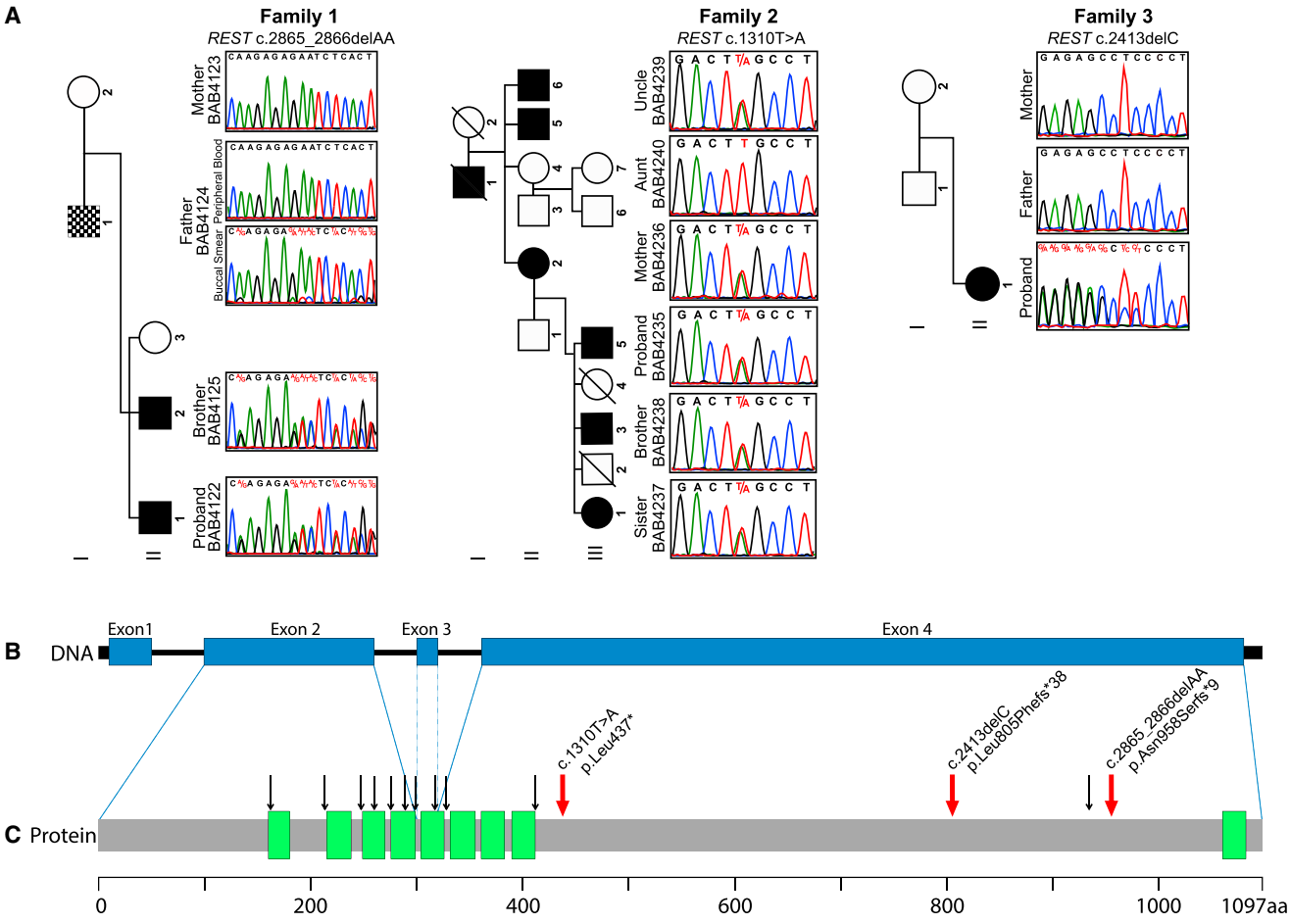

Figure 2. Segregation Analyses and Gene Variant Distributions in Three Families
(A) Sanger sequencing confirmed that the mother (I-2) is wild-type and both affected siblings (II-1 and II-2) are heterozygous carriers for the c.2865_2866delAA variant in family 1. The mildly affected father (I-1) was also found to have two wild-type alleles in his blood sample; however, Sanger sequencing of the buccal smear sample revealed evidence for subtle secondary peaks that begin at the position in the sequence of the frameshift variant observed in the two affected children. In families 2 and 3, Sanger sequencing confirmed that all affected individuals have the WES-identified heterozygous variant (c.1310T>A and c.2413delC, respectively) and the unaffected individuals are wild-type.
(B) Exonic structure and map position of variant alleles from three unrelated families; note all occur in last exon.
(C) Schematic representations of protein structure with indicated domains of REST mapped to gene exonic structure above showing localization of the identified variants in our study (red arrows) and previously reported variants in individuals with Wilms tumor (black arrows). Green rectangles indicate zinc-finger DNA-binding domains.

REST is located on chromosome 4q12, consists of 4 exons, and encodes a 1,097-amino acid protein[15,16] (Figures 2B and 2C). REST is a zinc finger protein that regulates gene expression throughout the body.[16,17] It has a critical function as a transcriptional repressor during embryonic development and neurogenesis so it is also called neuron-restrictive silencer element.[15] Additionally, REST has different roles in several cellular mechanisms, such as oncogenic and tumor-suppressor functions, hematopoietic, cardiac and osteoblast differentiation, and effects on chromatin-modifying enzymes.[17–21]

The pathophysiologic mechanisms underlying HGF remain enigmatic; however, excessive accumulation of extracellular matrix components, particularly collagen type I, seem to contribute to the clinical-pathologic manifestations.[22–24] In a comparative study performed on normal gingiva and HGF fibroblasts, Martelli-Junior et al. showed that the expression and production of type I collagen is significantly higher in fibroblasts from HGF than from normal gingiva.[24] They also demonstrated that addition of TGF-β1 and IL-6, which are produced in greater amounts by HGF fibroblasts, promoted an increase in type 1 collagen. Type 1 collagen is the major component of the extracellular matrix which is produced in principal by fibroblasts and TGF-β and IL-6 have crucial roles in promoting the synthesis of the collagen together with other growth factors. Pathway analysis performed by Kong et al. showed that putative REST target genes were widely involved in TGF-β signaling and in the same study they showed that the inhibition of REST upregulates the TGF-β signaling pathway.[25] Another study implemented on neurendocrine differentiation in prostate cancer cells

revealed that knockdown of REST activates the IL-6-induced autopaghy pathway.[26] The authors also showed that IL-6-induced neuronal cell morphology changes in prostate cancer cells were significantly inhibited when there was REST overexpression. This finding was confirmed in a subsequent study by showing that the overexpression of exogenous REST abrogates IL-6-induced neuroendocrine differentiation in prostate cancer cells.[27] Taken together we can speculate that truncating mutations in *REST* may lead to overexpression of TGF-β and IL-6 that increase collagen type 1 synthesis in gingival tissue and results in GF. To show the profibrotic effects of TGFβ in gingival fibroblasts, Thompson et al. tested the effect of a specific pharmacological inhibitor of TGFβ type 1 receptor on the ability of TGFβ to induce connective tissue growth factor (which is a marker and mediator of fibrosis, not normally expressed in gingival fibroblasts, but is induced by the potent profibrotic cytokine TGFβ and is overexpressed in gingival fibrosis) in gingival fibroblasts.[28] Their results suggested that blocking TGFβ type 1 receptor may be useful in blocking the profibrotic effects of TGFβ in gingival fibroblasts.

Dysregulation of REST has been implicated in diverse diseases such as Alzheimer disease (MIM: 104300), Huntington disease (MIM: 143100), and Down syndrome (MIM: 190685) and also in several malignancies.[18,29–40] Recently, mutations in *REST* have been shown to predispose to Wilms tumor (MIM: 616806).[41] The identified *REST* mutations in individuals with Wilms tumor were clustered within the zinc-finger DNA-binding domain of REST (Figure 2C). Interestingly, only one out of eleven variants mapped outside of the DNA-binding domain; a nonsense mutation (c.2770C>T [p.Gln924*]) located in the final exon. The reported individual who carries this mutation is a girl who was diagnosed with Wilms tumor at 3.4 years of age and she has inherited the mutation from an unaffected parent. No other congenital abnormality has been reported for the affected child and no clinical data are available for the parent who transmitted the nonsense mutation. Lack of reported GF may be because the identified mutation in these individuals does not cause a gingival phenotype, or because the age of child is too early to observe GF and the parent was not examined for GF, or because the gingiva were not examined or noted on physical exam. Furthermore, Wilms tumor or any other malignancies were not detected to date in any of the individuals in our study.

There are also some truncating variants reported in the last exon of *REST* in publicly available databases (i.e., ExAC). *REST* is predicted to be intolerant to loss-of-function alleles with a 0.97 pLI score[42] in the ExAC database; however, since we do not have access to the samples from that database we cannot validate these putative truncating variants. The calling of indels in genome-wide sequencing remains a challenge because of poor mappability.[43] Therefore, frameshift variants identified in exome/genome sequencing need to be confirmed with Sanger sequencing or allele-specific cloning. Additionally, and most importantly, we lack clinical information to check whether those individuals with truncating mutations have GF or not. The data from our computational algorithm that enabled an NMD prediction approach suggest that these truncating variants identified in BAB4122 (II-1 in Figure 1A) and BAB4235 (III-5 in Figure 1B) likely escape from NMD consistent with a hypothesis that such alleles may act through either a dominant-negative (antimorphic) or gain-of-function (neomorphic) effect, rather than by a haploinsufficiency mechanism. Thus, the mutant transcripts may reduce the repressor function of REST on the collagen synthesis pathway that results in the accumulation of collagen in gingiva. Nevertheless, without a biological experiment specifically designed for testing the effects of various truncating variants of *REST*, the mechanism of GF-causing mutations remains unclear.

In this study, we describe three different heterozygous truncating variants, all of which are located in the final exon in *REST*, that cause autosomal-dominant HGF in three unrelated families. Interestingly, *REST* does not map to one of the four loci that have been previously associated with HGF. HGF is a rare disorder and sometimes, particularly the mild forms, can be overlooked during the physical examination and the affected individuals may not be referred to genetic clinics for diagnosis or further research studies. Therefore, we postulate that other genetic loci/genes remain to be identified. Further studies are needed to delineate the mutational spectrum and phenotype associations of both somatic and germline mutations in *REST* and to elucidate the role of REST in the molecular pathophysiology of HGF.

Accession Numbers

The accession numbers for the variants (c.2865_2866delAA, c.1310T>A, and c.2413delC) reported in this paper are ClinVar: SCV000579327, SCV000579328, and SCV000579329.

Supplemental Data

Supplemental Data include a Supplemental Note, two figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.ajhg.2017.06.006.

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J.R.L. has stock ownership in 23andMe and Lasergen, is a paid consultant for Regeneron Pharmaceuticals, and is a coinventor on multiple United States and European patents related to

molecular diagnostics for inherited neuropathies, eye diseases, and bacterial genomic fingerprinting. The Department of Molecular and Human Genetics at Baylor College of Medicine derives revenue from the chromosomal microarray analysis (CMA) and clinical exome sequencing offered in the Baylor Genetics Laboratory. M.T.C. and A.B. are employees of GeneDx.

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Web Resources

1000 Genomes, http://www.internationalgenome.org/
Atherosclerosis Risk in Communities Study (ARIC) Database, http://www2.cscc.unc.edu/aric/
Baylor Genetics Laboratory, http://bmgl.com/
cBioPortal for Cancer Genomics, http://www.cbioportal.org/
ClinVar, https://www.ncbi.nlm.nih.gov/clinvar/
ExAC Browser, http://exac.broadinstitute.org/
GenBank, http://www.ncbi.nlm.nih.gov/genbank/
GeneMatcher, https://genematcher.org/
IGV, http://www.broadinstitute.org/igv/
Likelihood Ratio Test, http://www.genetics.wustl.edu/jflab/lrt_query.html
MutationTaster, http://www.mutationtaster.org/
NHLBI Exome Sequencing Project (ESP) Exome Variant Server, http://evs.gs.washington.edu/EVS/
OMIM, http://www.omim.org/
PolyPhen-2, http://genetics.bwh.harvard.edu/pph2/
PROVEAN, http://provean.jcvi.org
UniProt, http://www.uniprot.org/

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