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Family-Based Genetic Association for Molar-Incisor Hypomineralization

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Key Words

Genetic association study · Genetic polymorphisms · Tooth hypomineralization

Abstract

Despite some evidence of genetic and environmental factors on molar-incisor hypomineralization (MIH), its aetiology remains unclear. This family-based genetic association study aimed more comprehensively to investigate the genetic carriage potentially involved in MIH development. DNA was obtained from buccal cells of 391 individuals who were birth family members of 101 Brazilian nuclear families. Sixty-three single nucleotide polymorphisms (SNPs) were investigated in 21 candidate genes related to amelogenesis using the TaqMan™ OpenArray™ Genotyping platform. All SNPs were genotyped in 165 birth family members unaffected by MIH, 96 with unknown MIH status and 130 affected individuals (50.7% with severe MIH). Association analysis was performed by the transmission/disequilibrium test (TDT), and statistical results were corrected using the false discovery rate. Significant results were obtained for SNPs rs7821494 (*FAM83H* gene, OR = 3.7; 95% CI = 1.75–7.78), rs34367704 (*AMBN* gene, OR = 2.7; 95% CI = 1.16–6.58), rs3789334 (*BMP2* gene, OR = 2.9; 95% CI = 1.34–6.35), rs6099486 (*BMP7* gene, OR = 2.2; 95% CI = 1.14–4.38), rs762642 (*BMP4* gene, OR = 2.3; 95% CI = 1.38–3.65), rs7664896 (*ENAM* gene, OR = 2.1; 95% CI = 1.19–3.51), rs1711399 (*MMP20* gene, OR = 0.4; 95% CI = 0.20–0.72), rs1711423 (*MMP20* gene, OR = 2.1; 95% CI = 1.18–3.61), rs2278163 (*DLX3* gene, OR = 2.8; 95% CI = 1.26–6.41), rs6996321 (*FGFR1* gene, OR = 2.7; 95% CI = 1.20–5.88), and rs5979395 (*AMELX* gene, OR = 11.7; 95% CI = 1.63–84.74). Through this family-based association study, we concluded that variations in genes related to amelogenesis were associated with the susceptibility to develop MIH. This result is in agreement with the multifactorial idea of the MIH aetiology, but further studies are necessary to investigate more thoroughly the factors that could influence MIH.

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The process of tooth formation originates from the interaction between the oral epithelium and the ectomesenchyme through a series of temporary and highly regulated events. This results in the differentiation of epithelial cells into ameloblasts, a process referred to as amelogenesis, which is a phase of enamel development. This process is very sensitive and includes developing a specific extracellular matrix, matrix processing, and controlling the microenvironment of the developing enamel tissue [He et al., 2010; Lacruz et al., 2012]. The genetic control of dental development represents a complex series of events, and occasional mutations in the genes coding enamel proteins may cause alterations that affect the molecular pathways. The consequence is the occurrence of a deficiency in the amount of enamel (hypoplasia), a change in the composition (hypomineralization), or a change in the enamel structure [Wright et al., 2015]. Therefore, amelogenesis is under strict genetic control, and even caries susceptibility can be affected by genetic variation [Simmer and Hu, 2001; Deeley et al., 2008; Vieira et al., 2008; Shimizu et al., 2012].

In the last several years, a particular pattern of hypomineralization that affects molars and incisors has gained importance. Molar-incisor hypomineralization (MIH) refers to demarcated, qualitative enamel defects of systemic origin, affecting one or more permanent molars with or without involvement of the incisor teeth [Weerheijm et al., 2001]. A recent literature review found 52 studies demonstrating a wide variation in the prevalence of MIH (2.9–44 %) [Elfrink et al., 2015]. In Brazil, there are data on the prevalence of 12.3% [Jeremias et al., 2013a] and 19.8% [da Costa-Silva et al., 2010] for different regions. One of the main characteristics of teeth affected by MIH is the greater porosity of the enamel, which can easily be fractured due to masticatory forces, leading to exposed dentin that may promote the development of caries. Therefore, MIH is associated with caries [Muratbegovic et al., 2007; Cho et al., 2008; Alaluusua, 2010].

In clinical practice, we have frequently observed patients affected by MIH and also their parents and siblings. However, the aetiology of MIH remains unclear [Alaluusua, 2010; Sönmez et al., 2013; Wuollet et al., 2014]. There are studies demonstrating a potential environmental contribution to MIH [Jan et al., 2007; Alaluusua, 2010; Souza et al., 2012; Loli et al., 2015; Oyedele et al., 2015] and other studies demonstrating evidence of the genetic influence on the occurrence of MIH [Jeremias et al., 2013b; Kühnisch et al., 2014]. Kuscu et al. [2013] mentioned that MIH is a multifactorial disturbance, but the specific environmental factors and the genetic contribution are still not completely understood. Therefore, we evaluated the presence of genetic association in 63 single nucleotide polymorphisms (SNPs) of 21 candidate genes related to amelogenesis in Brazilian nuclear families affected by MIH using a family-based genetic association approach.

Materials and Methods

Subject Screening and Sample Collection

A total of 101 eligible nuclear families were enrolled from the Pedodontics Clinics of São Paulo State University and from day care facilities in Araraquara, Brazil. All birth family members (n = 391 individuals) enrolled in this study gave their informed consent/assent to participate in this study, which was approved by the Research Ethics Committee of the FOAr-UNESP (protocol No. 45/10) according to the guidelines of the Declaration of Helsinki.

The exclusion criteria included having evidence of enamel formation linked to a condition such as amelogenesis imperfecta or dental fluorosis or to the use of a fixed appliance. Calibrated examiners (two paediatric dentists/researchers) carried out the clinical examination in all birth family members to investigate enamel defects and dental caries. Clinical examinations were performed with the use of a flashlight and a mouth mirror. Gauze was used to dry and clean the teeth prior to examination (researcher L.S.-P. was the calibrator for F.J. and C.M.B.F). Examination calibrations were performed according to the following protocol. First, the calibrator presented the examiners with the criteria for MIH detection, showed pictures of several situations that would be observed during the examination, and discussed each of these situations in a session that lasted 1–2 h. Next, the calibrator and examiners examined 10–20 subjects and discussed each case. The intra-examiner agreement was assessed by a second clinical examination in 10% of the sample after 2 weeks, with a kappa of 1.0. The MIH phenotype (enamel opacity, enamel breakdown, and atypical restoration) was based on clinical findings defined by the criteria of the European Academy of Paediatric Dentistry (EAPD) [Weerheijm et al., 2003].

Buccal epithelial cells from the subjects were obtained using 3 ml of 3% glucose mouthwash for 2 min. DNA was extracted utilizing the PureLink Genomic DNA Mini Kit (Invitrogen™, Carlsbad, Calif., USA) according to the manufacturer's instructions. The purity and concentration of the samples were checked using a NanoVue™ Spectrophotometer (GE Healthcare, Little Chalfont, UK).

Genotyping

We used the SNP Browser 4.0 software (Applied Biosystems, Foster City, Calif., USA) to select the 63 SNPs among 21 genes involved in enamel formation (table 1). The SNPs were genotyped using the TaqMan® OpenArray™ Genotyping System and the TaqMan™ Genotyper software version 1.0.1. (Applied Biosystems). The quality value of a data point's genotype was determined by a threshold above 0.95.

Statistical Analysis

Genotyped data and minor allele frequency (minimum allele frequency) of the SNPs were estimated by the JINGLEFIX program [Secolin et al., 2008]. Mendelian inconsistencies were evaluated by

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Table 1. Genes analysed according to markers and alleles

Gene	SNP (rs)	Allele	Gene	SNP (rs)	Allele
MMP20	2280211	CT	Amelotin	17676820	CT
MMP20	1784410	GT	Amelotin	13151614	CG
MMP20	1711399	AC	Amelotin	17149007	CT
MMP20	1711441	AC	Distal-less 3 homeobox	2278163	CT
MMP20	1784438	AG	Distal-less 3 homeobox	3891034	AG
MMP20	1711422	AG	Kallikrein	2235091	CT
MMP20	1784423	CT	Kallikrein	198968	AG
MMP20	1784441	CT	Bone morphogenetic protein-2	3789334	AG
MMP20	1784440	AG	Bone morphogenetic protein-2	235767	GT
MMP20	1711423	AC	Bone morphogenetic protein-2	15705	AG
Tuftelin	4970956	AG	Bone morphogenetic protein-2	1005464	AG
Tuftelin	7526319	CT	Bone morphogenetic protein-4	17563	CT
Tuftelin	11204848	CT	Bone morphogenetic protein-4	762642	GT
Tuftelin	3790506	CT	Bone morphogenetic protein-4	2071047	CT
Family with sequence similarity 83	7821494	CG	Bone morphogenetic protein-7	6099486	CT
Family with sequence similarity 83	7463064	CT	Bone morphogenetic protein-7	927836	CT
Family with sequence similarity 83	12681370	AG	Bone morphogenetic protein-7	12479955	AG
Dentin sialophosphoprotein	2615487	CT	Bone morphogenetic protein-7	230191	CT
Dentin sialophosphoprotein	3750025	CT	Metallopeptidase inhibitor 2	2376999	CT
Dentin sialophosphoprotein	2615489	AG	Metallopeptidase inhibitor 2	7218729	CG
Amelogenin	6530435	AG	Fibroblast growth factor receptor 1	6987534	CG
Amelogenin	6654939	CT	Fibroblast growth factor receptor 1	6996321	AG
Amelogenin	5979395	AG	Fibroblast growth factor receptor 2	4752566	GT
Amelogenin	946252	AG	Fibroblast growth factor receptor 2	2981427	AG
Amelogenin	5934996	AT	Fibroblast growth factor receptor 2	1078806	CT
Amelogenin	17878486	CT	Fibroblast growth factor 1	34010	AC
Enamelin	2609426	AG	Fibroblast growth factor 2	1048201	CT
Enamelin	7664896	CG	Parathyroid hormone	307253	CT
Enamelin	1055660	CT	Parathyroid hormone receptor 1	7652849	CT
Ameloblastin	34367704	AG	Parathyroid hormone receptor 1	6442307	AC
Ameloblastin	4694075	CT	Transforming growth factor, beta 1	11568785	AG
			Transforming growth factor, beta 1	4803455	AC

PEDCHECK software [O'Connell and Weeks, 1998]. The Hardy-Weinberg equilibrium and the degree of linkage disequilibrium between SNPs were evaluated by the HAPLOVIEW program [Barrett et al., 2005].

Family-based association analysis was performed by the transmission/disequilibrium test (TDT) using the UNPHASED program [Dudbridge, 2008], and statistical results were adjusted for multiple testing using the false discovery rate. The statistical power (1-β) of the sample was evaluated using the TDT power calculator 1.2.1 [Chen and Deng, 2001], including the following parameters: $1-\beta > 0.8$, corrected type I error = 0.05, and prevalence = 12.3% [Jeremias et al., 2013a].

Table 2. Proportion of affected individuals per nuclear family

MIH-affected individuals per family, n	Nuclear families, n (%)
1	79 (78.2)
2	17 (16.8)
3	3 (3.0)
4	2 (2.0)
Total	101

Results

There were 79 (78.2%) nuclear families with only 1 MIH-affected individual and 22 (21.8%) with 2 or more MIH-affected individuals, including siblings and parents (table 2). In total, there were 165 birth family members unaffected by MIH, 96 with unknown MIH status and 130 MIH-affected individuals. Of the individuals evaluated, 49.6% were male (mean age 10 years; DMFT = 1.8). Among the affected individuals (n = 130), 50.7% were

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Table 3. TDT analysis of the SNPs in MIH families

SNP (rs)	Informative trios, n	Mendelian errors, n	Minor allele frequency	Alleles	Hardy-Weinberg p value	TDT p value	FDR-corrected p values
3790506	48	0	0.279	T:C	0.099	0.6168	0.7771
4970956	48	0	0.268	G:A	0.372	0.0185	0.0685
7526319	48	0	0.406	T:C	0.091	0.1099	0.2387
11204848	47	0	0.372	T:C	0.087	0.9093	0.9709
198968	48	0	0.191	G:A	1.000	0.8788	0.9709
2235091	48	0	0.312	T:C	0.132	0.7097	0.8279
7821494	47	0	0.392	C:G	*0.467*	*0.0000*	*0.0000*
12681370	48	0	0.275	G:A	0.885	0.4101	0.6151
7463064	46	0	0.339	T:C	0.053	1.0000	1.0000
34367704	48	0	0.138	G:A	*0.389*	*0.0102*	*0.0457*
4694075	48	0	0.422	T:C	0.000	0.1016	0.2387
15705	47	0	0.460	G:A	0.009	0.0000	0.0000
235767	46	0	0.345	T:G	1.000	0.0823	0.2257
3789334	47	0	0.198	G:A	*0.526*	*0.0016*	*0.0144*
1005464	46	0	0.061	G:A	1.000	0.5257	0.7199
927836	46	0	0.095	T:C	1.000	0.8083	0.9093
230191	45	0	0.345	T:C	0.067	0.2618	0.4340
12479955	44	0	0.140	G:A	1.000	0.1090	0.2387
6099486	48	0	0.181	T:C	*0.476*	*0.0109*	*0.0457*
17563	48	0	0.460	T:C	0.538	0.4726	0.6616
762642	47	0	0.356	G:T	*0.058*	*0.0006*	*0.0075*
2071047	48	0	0.379	T:C	0.675	0.6615	0.8171
1055660	47	0	0.013	T:C	1.000	1.0000	1.0000
7664896	47	0	0.490	G:C	*0.534*	*0.0058*	*0.0332*
2609426	48	0	0.279	A:G	0.003	0.4726	0.6616
1784410	46	0	0.297	T:G	0.860	0.2227	0.3897
1711399	47	0	0.312	C:A	*1.000*	*0.0013*	*0.0136*
1711441	48	0	0.356	C:A	0.058	0.0918	0.2387
1784441	48	0	0.131	T:C	0.494	0.0824	0.2257
1784440	48	0	0.319	G:A	0.179	0.1361	0.2858
1784423	47	0	0.399	T:C	0.006	0.0124	0.0488
1711422	48	0	0.379	G:A	0.530	0.2160	0.3897
2280211	47	0	0.331	T:C	0.880	0.0232	0.0812
1784438	47	0	0.226	G:A	0.149	0.3253	0.5254
1711423	48	0	0.383	C:A	*0.268*	*0.0069*	*0.0344*
34010	45	0	0.003	C:A	1.000	1.0000	1.0000
1078806	47	0	0.476	T:C	0.609	0.3605	0.5677
2981427	47	0	0.419	G:A	0.025	0.1063	0.2387
4752566	48	0	0.436	G:T	0.362	0.5468	0.7329
2278163	47	0	0.213	T:C	*0.162*	*0.0036*	*0.0252*
3891034	48	0	0.477	G:A	0.166	0.7008	0.8279
11568785	46	0	0.133	G:A	0.962	0.2187	0.3897
1048201	47	0	0.372	C:T	0.153	0.1633	0.3215
6442307	47	0	0.423	A:C	0.002	0.7388	0.8462
7652849	48	0	0.493	T:C	0.836	0.5927	0.7620
4803455	47	0	0.269	C:A	0.008	0.1001	0.2387
6987534	46	0	0.361	G:C	0.001	0.2014	0.3844
6996321	47	0	0.186	G:A	*0.409*	*0.0071*	*0.0344*
307253	47	0	0.114	T:C	1.000	0.6826	0.8270
2376999	48	0	0.490	T:C	0.778	0.8981	0.9709
7218729	47	0	0.405	G:C	0.021	0.2366	0.4028
5934996	47	0	0.483	A:T	1.000	0.4220	0.6182
946252	46	0	0.443	A:G	0.033	0.0035	0.0252
5979395	44	0	0.104	G:A	*0.359*	*0.0001*	*0.0015*

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Table 3 (continued)

SNP (rs)	Informative trios, n	Mendelian errors, n	Minor allele frequency	Alleles	Hardy-Weinberg p value	TDT p value	FDR-corrected p values
6654939	48	0	0.493	T:C	0.000	0.0047	0.0296
6530435	47	0	0.091	G:A	0.039	0.0000	0.0000
17878486	48	0	0.168	T:C	0.339	1.0000	1.0000
17149007	46	1	0.306	C:T	0.655	0.3952	0.6072
13151614	47	0	0.302	G:C	0.243	0.0403	0.1304
17676820	46	0	0.487	C:T	0.063	0.1552	0.3154
3750025	48	0	0.017	T:C	1.000	0.0414	0.1304
2615489	46	3	0.397	A:G	0.059	0.0633	0.1899
2615487	48	0	0.372	T:C	0.037	0.5787	0.7595

p values were corrected using the false discovery rate (FDR). Values in italics show statistically significant differences ($p < 0.05$).

diagnosed with severe MIH (when the child had at least one tooth with structural loss).

Table 3 shows the results of the TDT analysis. The distribution of SNP genotypes followed the Hardy-Weinberg equilibrium. We found significant results for SNPs rs7821494 (*FAM83H* gene, p = 0.00004, OR = 3.7; 95% CI = 1.75–7.78), rs34367704 (*AMBN* gene, p = 0.0457, OR = 2.7; 95% CI = 1.16–6.58), rs3789334 (*BMP2* gene, p = 0.0144, OR = 2.9; 95% CI = 1.34–6.35), rs6099486 (*BMP7* gene, p = 0.0457, OR = 2.2; 95% CI = 1.14–4.38), rs762642 (*BMP4* gene, p = 0.0075, OR = 2.3; 95% CI = 1.38–3.65), rs7664896 (*ENAM* gene, p = 0.0332, OR = 2.1; 95% CI = 1.19–3.51), rs1711399 (*MMP20* gene, p = 0.0136, OR = 0.4; 95% CI = 0.20–0.72), rs1711423 (*MMP20* gene, p = 0.0344, OR = 2.1; 95% CI = 1.18–3.61), rs2278163 (*DLX3* gene, p = 0.0252, OR = 2.8; 95% CI = 1.26–6.41), rs6996321 (*FGFR1* gene, p = 0.0344, OR = 2.7; 95% CI = 1.20–5.88), and rs5979395 (*AMELX* gene, p = 0.0015, OR = 11.7; 95% CI = 1.63–84.74). Regarding rs5979395, we observed that 97% of rs5979395*G alleles were transmitted to MIH-affected individuals, whereas only 3% of rs5979395*A alleles were transmitted to unaffected individuals.

Discussion

This is the first family-based association study conducted regarding MIH. In summary, family-based association designs aimed to avoid the potential confounding effects of population stratification by using the parents or unaffected siblings as the controls for the case patients. The statistical considerations for family-based studies differ from those of population-based investigations. Individuals within the same family are likely to be more similar to one another than are individuals from different families. This phenomenon is referred to in statistics as clustering and implies a within-family correlation. The idea is that there is something unmeasurable (latent), such as diet or underlying biological make-up, that makes people from the same family more alike than people across families. As a result, the trait under investigation is more highly correlated among individuals within the same family. Accounting for the potential within-cluster correlation in the statistical analysis of family-based data is essential to making valid inferences in these settings [Foulkes, 2009].

Genetic associations between SNP rs3790506 *(TUFT1)* and SNP rs946252 *(AMELX)* and MIH have previously been investigated by our group using a population-based association design [Jeremias et al., 2013b], but we did not find an association between these SNPs and MIH. Since those SNPs reside in important genes related to amelogenesis [Simmer and Hu, 2001; Deutsch et al., 2002; Stephanopoulos et al., 2005], we evaluated them again in this present study, enrolling a family-based Brazilian sample (391 individuals from 101 nuclear families); however, no significant associations were found. Thereby, we evaluated an additional 63 SNPs in 21 candidate genes related to MIH because they exert some function in any of the pre-secretory, secretory, transitional, or maturation stages of amelogenesis and because the entire amelogenesis process is under genetic control [Simmer and Hu, 2001].

In the following sections, we will discuss our present genetic findings and the rationale for investigating each gene in this study. We found an association between SNP

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rs5979395, in the *AMELX* (Xq22) gene, and MIH (p = 0.006, OR = 11.7). In addition, 97% of MIH-affected individuals carry the rs5979395*G allele. Because approximately 5–10% of all amelogenesis imperfecta cases are X-linked, it is predictable to find a mutation in the *AMELX* gene [Bäckman, 1988]. Our study demonstrates that genetic variation in the *AMELX* gene is associated not only with amelogenesis imperfecta but also with MIH. This gene is fundamental for amelogenesis, which codifies amelogenin, the main protein of dental enamel secreted by ameloblasts during the secretion stage of amelogenesis [Stephanopoulos et al., 2005; Chan et al., 2011].

As ameloblasts differentiate, they deposit in the secretory stage important non-amelogenin enamel matrix proteins, including enamelin *(ENAM)* and ameloblastin *(AMBN)*, whose genes are located in the long arm of chromosome 4 (4q13) near other genes associated with mineralized tissues, such as osteopontin, bone sialoprotein, bone morphogenetic protein 3, and dentin sialophosphoprotein (4q22) [MacDougall et al., 1997]. Here, the TDT analysis showed a significant association with MIH of the SNPs in the *ENAM* (rs7664896) and *AMBN* (rs34367704). Interestingly, *AMBN* is also expressed in the maturation stage [Winter and Brook, 1975], but other important genes such as amelotin (*AMTN*, 4q13), which is specifically expressed in maturation-stage ameloblasts [Iwasaki et al., 2005], was not associated with MIH in this study. Tuftelin (*TUFT1*, 1q21) has been suggested to play an important role during enamel development and mineralization [Deutsch et al., 2002]. Kallikrein-related peptidase 4 (*KLK4*, 19q13) is produced during the maturation stage to further process the remaining organic matrix [Simmer and Hu, 2001]. Moreover, we decided to include in this comprehensive genetic analysis genes encoding bone morphogenetic proteins, such as *BMP2* (20p12), *BMP4* (14q22), and *BMP7* (20q13), because they are expressed in the pre-ameloblasts and ameloblasts (http://honeybee.helsinki.fi/toothexp). We found an association between MIH and SNP rs3789334 in the *BMP2*, SNP rs762642 in the *BMP4*, and SNP rs6099486 in the *BMP7* gene. We highlight the *BMP4* gene which encodes a protein with a vital regulatory function throughout development in mesoderm induction, tooth development, limb formation, bone induction, and fracture repair [Bakrania et al., 2008]. BMP4 knock-out mice showed decreased mature odontoblast differentiation. The mechanism that explains this finding is that the absence of the *BMP4* gene decreases not only the BMP signalling but also the expression of three key transcription factors: Dlx3, Dlx5, and osterix. Distal-less 3 (*DLX3*, 17q21) is highly expressed in ameloblasts and controls many of the downstream genes involved in amelogenesis, such as ameloblastin and amelogenin [Gluhak-Heinrich et al., 2010]. Here we found that the SNP rs2278163 in the *DLX3* gene was associated with MIH. BMP signalling, as well as Dlx3 and amelogenin expression, is also indirectly reduced in the ameloblasts and odontoblasts of BMP4 knock-out mice. This supports a key paracrine or endocrine role of odontoblasts derived from BMP4 postnatally on the proper amelogenesis and formation of the enamel [Gluhak-Heinrich et al., 2010]. Interestingly, our study is the first to identify the association of genetic variations in the *BMP4* gene with MIH. A genome-wide association study identified SNP rs13058467 (located near the *SCUBE1* gene) as being associated with MIH [Kühnisch et al., 2014]. Even though that study was underpowered, the authors mentioned that the *SCUBE1 g*ene, normally involved in vascular biology, can directly bind to BMP in the epithelial and mesenchymal regions of the developing tooth [Tu et al., 2008], and SCUBE proteins may negatively regulate BMP activity [Aberg et al., 1997; Helder et al., 1998].

The investigated genes fibroblast growth factor 1 (*FGF1*, 5q31), fibroblast growth factor receptor 1 (*FGFR1,* 8p11), fibroblast growth factor 2 (*FGF2,* 4q27), its receptor (*FGFR2,* 10q26), parathyroid hormone (*PTH,* 11p15), its receptor (*PTHR1,* 3p21), and the transforming growth factor, β-1 (*TGFB1,* 19q13) were also included because they are expressed in the differentiation/secretion stages of the incisor/molar ameloblasts of rodents (http://honeybee.helsinki.fi/toothexp). Considering these genes, the only one associated with MIH was the *FGFR1* gene SNP rs6996321. In addition, we utilized a wide range of amelogenesis gene expression data in that site, and we noted that this information is in agreement with the literature because amelogenesis is characterized by a dynamic process occurring in dental tissue development, including cellular, biochemical, genetic, and epigenetic changes [Deutsch et al., 2002; Stephanopoulos et al., 2005; Chan et al., 2011].

The removal of amelogenins during maturation is a critical step in enamel mineralization. During tooth development, proteases, such as the matrix metalloproteinase-20 (MMP20), also named enamelysin (*MMP20*, 11q22), are secreted by ameloblasts and cleave the enamel proteins. MMP20 is a tooth-specific MMP secreted into the enamel matrix during the secretory and transition stages of enamel development [Turk et al., 2006] and is essential for the formation of a properly hardened enamel layer [Shin et al., 2014]. Demonstrating the genetic association of this gene with MIH, 2 SNPs were found to be associated with this enamel defect (rs 1711399 and rs 1711423). The tissue inhibitor of metalloproteinase 2 (*TIMP2*, 17q25) is a

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natural inhibitor of the matrix metalloproteinases, a group of peptidases involved in the degradation of the extracellular matrix [Noda et al., 2003]. *TIMP2* is also expressed in the secretory stage of molar mouse ameloblasts (http://honeybee.helsinki.fi/toothexp).

Family with sequence similarity 83, member H (*FAM83H*, 8q24.3) encodes an intracellular protein of unknown function that appears to be associated with the Golgi apparatus or trans-Golgi network [Ding et al., 2009] and is most strongly expressed by pre-ameloblasts [Lee et al., 2009]. Based on its expression pattern, Lee et al. [2009] suggested that *FAM83H* may be involved in the differentiation of pre-ameloblasts into functional ameloblasts and in enamel matrix calcification. The FAM83H protein is required for proper dental enamel calcification [Kim et al., 2008]. Recently, Zhang et al. [2015], showed that FAM83H could influence enamel biomineralization and dentine formation, and they reviewed studies that identified different mutations in the *FAM83H* gene associated with autosomal dominant hypocalcified amelogenesis imperfecta (ADHCAI) [Zhang et al., 2015]. There are at least 14 different registered mutations in the *FAM83H* gene associated with ADHCAI (http://omim.org/entry/611927), and Kim et al. [2008] was the first group to observe this. Interestingly, mutations in the *FAM83H* gene are not the only factors that can influence enamel formation. Polymorphisms might have a slight effect on amelogenesis, mainly regarding the enamel mineralization/maturation, because we have demonstrated here the significant association of SNP rs7821494 in the *FAM83H* gene with the genetic predisposition to MIH. Although our results reinforce the evidence regarding the influence of the *FAM83H* gene on amelogenesis, the mechanisms and functions of the FAM83H protein during enamel and dentine formation remain unclear [Zhang et al., 2015].

A potential limitation of our study resides in the accuracy of the diagnosis of the presence/absence of enamel defects, which is more difficult in adult subjects because developmental enamel defects may have been masked by restorations and remineralizing agents. However, the differences found here between MIH-affected patients and unaffected birth family members were significant enough to overcome a potential MIH misdiagnosis. Moreover, the significant genetic factors predisposing subjects to MIH found in this study could be inherent to the population studied. It is necessary to extrapolate these results to other populations because the Brazilian population is an interethnic admixture of Europeans, Africans, and autochthonous Amerindians, thus forming one of the most heterogeneous populations in the world [Alves-Silva et al., 2000]. Obviously, future studies enrolling different ethnic populations affected by MIH should be conducted. Not surprisingly, it is possible that other genes could be identified in different ethnic populations, as observed for other multifactorial diseases, mainly because of the influence of different environmental factors.

Our currents results support the idea that MIH is a multifactorial disturbance [Kuscu et al., 2013] because different genes can influence the occurrence of MIH. However, there is evidence of participating environmental factors [Loli et al., 2015; Oyedele et al., 2015]. These might modify the expression of any genes related to tooth formation at all amelogenesis stages. Moreover, there is substantial evidence supporting the involvement of epigenetic components in defining human phenotypic variations. Epigenetics describe the way in which gene-environment and gene-gene interactions shape a phenotype during development. Currently, epigenetics describe alterations in genomic function, mainly mitotically heritable changes in gene expression, that occur through reversible chemical modifications to the structure of chromatin without altering the DNA sequence [Godfrey et al., 2007].

In conclusion, we showed evidence of the genetic influence on MIH. This result is in agreement with the multifactorial idea of the MIH aetiology, but to prove this, further studies enrolling larger, well-diagnosed and different ethnic populations are necessary to expand the investigation of the genetic and environmental factors as well as the gene-environment interactions that might influence the occurrence of MIH.

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Author Contributions

Data analysis: F.J., R.S., R.M.S.-C. Study design: F.J., R.S., C.V.M.-M., L.S.-P., R.M.S.-C. Obtained funding: F.J., R.M.S.-C., L.S.-P. Manuscript writing: F.J., R.S., R.M.S.-C. Data collection: F.J., R.A.G.P., M.R., D.G.B., J.F.S., C.M.B.F. DNA manipulation/genotyping: F.J., M.R., R.S., R.M.S.-C., L.S.F. Revised and reviewed the paper: F.J., R.A.G.P., J.F.S., C.M.B.F., M.R., L.S.F., D.G.B., R.C.L.C., R.S., C.V.M.-M., L.S.-P., R.M.S.-C.

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Disclosure Statement

The authors certify that they have no commercial or associative interests that represent a conflict of interest in connection with this paper.

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