Mutational characterization of the P3H1/CRTAP/CypB complex in recessive osteogenesis imperfecta

C. Barbirato[1,2], M. Trancozo[1,2], M.G. Almeida[1,2], L.S. Almeida[1,2], T.O. Santos[1], J.C.G. Duarte[1], M.R.G.O. Rebouças[3], V. Sipolatti[3], V.R.R. Nunes[3] and F. Paula[1,2]

[1]Núcleo de Genética Humana e Molecular, Departamento de Ciências Biológicas, Centro de Ciências Humanas e Naturais, Universidade Federal do Espírito Santo, Vitória, ES, Brasil
[2]Programa de Pós-Graduação em Biotecnologia, Rede Nordeste de Biotecnologia, Centro de Ciências da Saúde, Universidade Federal do Espírito Santo, Vitória, ES, Brasil
[3]Hospital Infantil Nossa Senhora da Glória, Vitória, ES, Brasil

Corresponding author: C. Barbirato
E-mail: clarabarbirato@yahoo.com.br

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ABSTRACT. Osteogenesis imperfecta (OI) is a genetic disease characterized by bone deformities and fractures. Most cases are caused by autosomal dominant mutations in the type I collagen genes *COL1A1* and *COL1A2*; however, an increasing number of recessive mutations in other genes have been reported. The *LEPRE1*, *CRTAP*, and *PPIB* genes encode proteins that form the P3H1/CRTAP/CypB complex, which is responsible for posttranslational modifications of type I collagen. In general, mutations in these genes lead to severe and lethal phenotypes of recessive OI. Here, we describe sixteen genetic variations detected in *LEPRE1*, *CRTAP*, and *PPIB* from 25 Brazilian patients with OI. Samples were screened for mutations on single-strand conformation polymorphism gels and variants were determined by automated sequencing. Seven variants were detected

in patients but were absent in control samples. *LEPRE1* contained the highest number of variants, including the previously described West African allele (c.1080+1G>T) found in one patient with severe OI as well as a previously undescribed p.Trp675Leu change that is predicted to be disease causing. In *CRTAP*, one patient carried the c.558A>G homozygous mutation, predicted as disease causing through alteration of a splice site. Genetic variations detected in the *PPIB* gene are probably not pathogenic due to their localization or because of their synonymous effect. This study enhances our knowledge about the mutational pattern of the *LEPRE1*, *CRTAP*, and *PPIB* genes. In addition, the results strengthen the proposition that *LEPRE1* should be the first gene analyzed in mutation detection studies in patients with recessive OI.

Key words: Osteogenesis imperfecta; Mutations; LEPRE1; CRTAP; PPIB

INTRODUCTION

Osteogenesis imperfecta (OI) is a heterogeneous genetic disorder typically characterized by bone fragility and deformity, recurrent fractures, blue sclera, short stature, and dentinogenesis imperfecta. OI is traditionally classified into types I (mild), II (lethal), III (severe), and IV (moderate) based on clinical and radiological findings (Sillence et al., 1979). However, due to the observation of unique characteristics and the discovery of new genes, recent studies have proposed to classify the disease according to the phenotype and location of the mutations (Forlino et al., 2011; Marini and Blissett, 2013). In general, however, the traditional classification is still used when the patients are not analyzed for genetic mutations.

The majority of cases of OI are caused by autosomal dominant mutations in the *COL1A1* or *COL1A2* genes that code for type I collagen chains, an important structural protein of connective tissues such as bone, skin tendons, and ligaments (Barsh and Byers, 1981; Gajko-Galicka, 2002). In 2012, an autosomal dominant mutation was described in *IFITM5*, which codes for an osteoblast-specific transmembrane protein that might be related to bone mineralization (Moffatt et al., 2008; Cho et al., 2012; Semler at al., 2012).

In addition, over the past few years, a large number of genes (*LEPRE1*, *CRTAP*, *PPIB*, *SERPINH1*, *FKBP10*, *SERPINF1*, *SP7*, *PLOD2*, *BMP1*, *TMEM38B*, *WNT1*, and *CREB3L1*) have been found to be related to recessive forms of OI (Morello et al., 2006; Cabral et al., 2007; van Dijk et al., 2009; Alanay et al., 2010; Christiansen et al., 2010; Lapunzina et al., 2010; Becker et al., 2011; Martínez-Glez et al., 2011; Puig-Hervás et al., 2012; Shaheen et al., 2012; Fahiminiya et al., 2013; Symoens et al., 2013). In general, mutations in these genes affect processes such as type I collagen posttranslational modification and secretion or bone mineralization and result in severe and lethal OI forms (Dalgleish, 1998).

Most cases of recessive OI are caused by mutations in *LEPRE1*, *CRTAP*, and *PPIB*, which encode prolyl 3-hydroxylase-1 (P3H1), cartilage-associated protein (*CRTAP*), and cyclophilin B (CypB), respectively. Together, these proteins form a molecular complex that is responsible for the 3-hydroxylation of a proline residue at position 986 of the type I collagen α1 chains. Prolyl 3-hydroxylation is one of the posttranslational modifications of type I collagen chains that contribute to the proper folding, stability, and secretion of the molecules (Vranka et al., 2004; Cabral et al., 2007).

In addition to prolyl 3-hydroxylation, the P3H1/CRTAP/CypB complex also functions as a PPIase, being the CypB a peptidyl-prolyl cis-trans isomerase for type I collagen, and as a chaperone, preventing type I collagen chains from forming premature aggregates in the endoplasmic reticulum (Ishikawa et al., 2009).

Despite these recent advances, information regarding the profile of mutations for the majority of genes associated with recessive OI is scarce. The mutational characterization and the description of non-pathogenic polymorphisms in genes related to OI are crucial to improve the accuracy of molecular diagnosis and of genetic counseling for OI families. In the present study, we describe sixteen genetic variations in *LEPRE1, CRTAP*, and *PPIB*; among these are both polymorphisms and pathogenic mutations, including five novel changes and three mutations predicted as disease causing.

MATERIAL AND METHODS

Samples

Clinical data and peripheral blood samples of 25 unrelated patients with OI were collected at the Nossa Senhora da Glória Children's Hospital (HINSG), located in Vitória, a Southeastern city in Brazil. The probands were clinically diagnosed with OI according to the traditional classification (Sillence et al., 1979). Of these, 12 patients had mild symptoms, seven had severe OI, and six had the moderate form of the disease. In addition, 100 unaffected individuals from the same population voluntarily participated as control samples for the polymorphism analysis. When available, samples from the parents and relatives of the patients were also collected and analyzed.

This study was approved by the Research Ethics Committee of the Nossa Senhora da Glória Children's Hospital and written informed consent was obtained from all participants.

Molecular analysis

DNA was extracted from peripheral blood cells using the methodology described by Miller et al. (1988). Fragments containing the fifteen exons of *LEPRE1*, the seven exons of *CRTAP*, the five exons of *PPIB*, and their exon-intron boundaries were amplified by polymerase chain reaction (PCR) in an Applied Biosystems Veriti® 96-Well thermal cycler (Foster City, CA, USA). Primer sequences are presented in Tables 1-3. All fragments were shorter than 400 bp. PCR products were screened for mutations by single-strand conformation polymorphism (SSCP) on 5 and 7% polyacrylamide:5% glycerol gels and on 6% glycerol MDE (Cambrex Bio Science Rockland, Inc., Rockland, ME, USA) gels (Orita et al., 1989; Spinardi et al., 1991). Fragments showing abnormal migration patterns were sequenced to detect the variation in the patient DNA sequences when compared with the National Center for Biotechnology Information (NCBI) reference sequences NG_008123.1, NG_008122.1, and NG_012979.1 (for *LEPRE1*, *CRTAP*, and *PPIB*, respectively) and with mutations described in The Human Collagen Mutation Database (Dalgleish, 1998). Sanger sequencing for all exons of the *LEPRE1* gene was performed in two patients to find a possible second causative mutation.

In previous studies, the *COL1A1* and *COL1A2* genes of the patients analyzed in this study were analyzed through SSCP screening and automated sequencing and no mutations were detected.

Table 1. Primer sequences for PCR amplification of exons and exon/intron boundaries of the *LEPRE1* gene.

Exon	Fragment size (bp)	Primer	Primer sequences (5'-3')
1A	378	Forward	GGGCTGACTGAAAGGAAAAG
		Reverse	AGCGCAGGCGAAGGGCGC
1B	359	Forward	GGTCCTGAGCATGGAACG
		Reverse	CAACACTCCTCTCCCCAGAA
2*	389	Forward	CTCAGGGAGGCAGGGATT
		Reverse	AGTCCGGGAATTTGAGGTTAC
3*	390	Forward	CCCCTTTCCATCCATAACC
		Reverse	GAGTCCCCATTTATATTATCA
4	378	Forward	GGGGACTCTTCACCCTCATT
		Reverse	AAGCCAAACACCTTGAGGAA
5	297	Forward	GGTCCCTGGTGCTAGGATTT
		Reverse	GCCTACTCCCCTCTGCTACC
6	347	Forward	CTGGGGAACAGACAGAGAGC
		Reverse	CTCAGCCTCCAGCAAGTTTT
7*	239	Forward	GGCAGCTAGAAGGGACTTAGA
		Reverse	ATGCAGTTTCTTCAAGGTCCTC
8	394	Forward	AAGCACTTGAGGCTTCCTGA
		Reverse	TCCACTGAACTTGCACCCTA
9*	359	Forward	AAAATGACCTAGCGGGAGA
		Reverse	GGAAGAGGAAGGCGAAGGCTAC
10*	347	Forward	GTGGTAGCCTTCGCCTTC
		Reverse	AACATAACTCATCCTCGCTTCC
11	378	Forward	AGGTCCCTTCCACAACACAT
		Reverse	ACATTGGTTCCCCAACTGAA
12	332	Forward	CATTGGGCATTCCGTAGACT
		Reverse	CCAGTGTGTGTGTGCTAGGG
13	300	Forward	CACACAAAGCCACCCTCCT
		Reverse	CAAGGGTACCGCCCACTG
14	388	Forward	GGGAAGCCATACTGAAGAGC
		Reverse	GTGTCCCAAGTGCTCCTTTC
15A	384	Forward	CCTGGGAAGTAGCAGCTGAG
		Reverse	GCACCATGTAGAAGGCTGTG
15B	348	Forward	TGACTAGACCCATGGAGAGGA
		Reverse	AGACCTCTGGGACAGAATGG

*Primer sequences used to amplify exons 2, 3, 7, 9, and 10 were described by Baldridge et al., 2008.

Table 2. Primer sequences for PCR amplification of exons and exon/intron boundaries of the *CRTAP* gene.

Exon	Fragment size (bp)	Primer	Primer sequences (5'-3')
1A	399	Forward	CAGCTGGCGCCCAGATCCCC
		Reverse	GCAGCCGCAGGCTGATCTCC
1B	391	Forward	GCCGAGAGCGTGGGCTACCT
		Reverse	GAACTGGAGGGGCAACGCGG
2	392	Forward	CCTGGAAGTCATGGAACCTT
		Reverse	GCAGCTGCTTATGGAGAGAC
3	333	Forward	TGGTCTTGGTTCCCTTTGA
		Reverse	AGGCATGCAGGCAGAAAC
4	310	Forward	CTTTTTCATTTGGGCAGGAC
		Reverse	TGAACTCTCAACAACCGTAGC
5	294	Forward	TGGCCTTTTTGTTTAGAAGC
		Reverse	AAGGCACGAGGTAGTCTCCA
6	240	Forward	CCTCCCTCCTCCCAGTTCTA
		Reverse	AGGACTCAGCCTTCCAGTGA
7	238	Forward	TGATGGCCTCTCGGGATA
		Reverse	GGCTCTGAGGTATCAACAGC

Table 3. Primer sequences for PCR amplification of exons and exon/intron boundaries of the *PPIB* gene.

Exon	Fragment size (bp)	Primer	Primer sequences (5'-3')
1	259	Forward	CTTCCGGCCTCAGCTGTC
		Reverse	AGGAGGGGCTCAGCCAAG
2	283	Forward	TCTCCCATCCTCAGGTTAGC
		Reverse	CTCTGCAGGTCAGTTTGCTG
3	293	Forward	CGAGCAGGAGTTGTGGACTT
		Reverse	GAGCTGGGGAAGAAAGAGG
4	314	Forward	CGAATGTCTGCTTGGTTTGG
		Reverse	AATCCCCGGTGAGGATTG
5	306	Forward	TTCTCCTGAGCGGTGGAC
		Reverse	CTCCACCAGATGCCAGCAC

RESULTS

SSCP screening of 25 unrelated Brazilian patients with OI and sequencing of the abnormal fragments identified eleven genetic variations in *LEPRE1* (Figure 1), three in *CRTAP* (Figure 2), and two in *PPIB* (Figure 3).

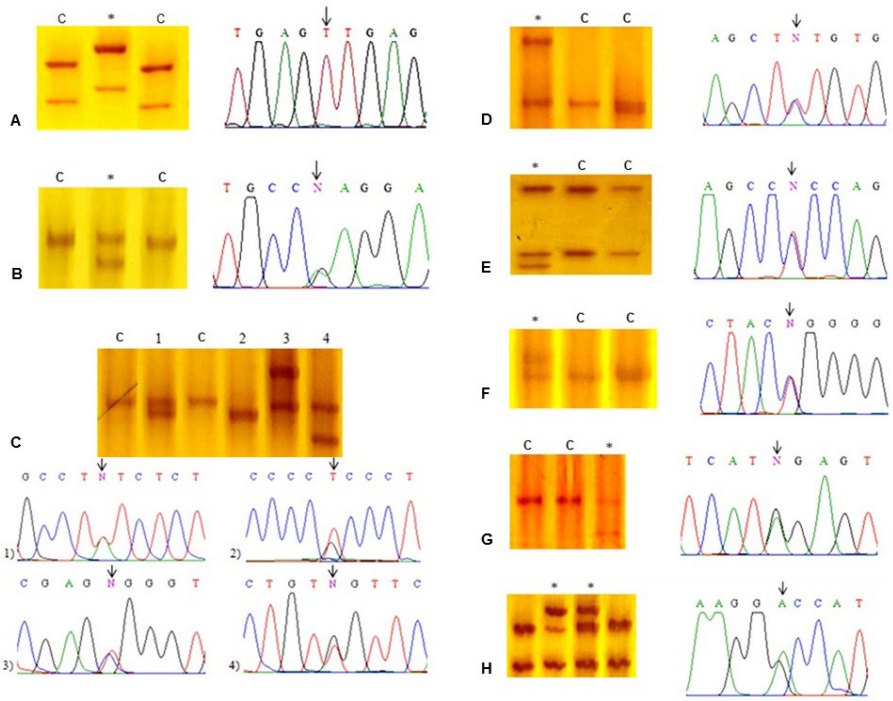

Figure 1. SSCP gels and sequencing of *LEPRE1* fragments. **A.** c.1080+1G>T detected in patient P1. **B.** c.1087A>G detected in patient P2. **C.** 1) c.2055+74T>A; 2) c.1915−20T>G; 3) c.2055+17C>T; 4) c.2024G>T/p.Trp675Leu detected in patient P3. **D.** c.1720+52C>T in patient P4. **E.** c.1812C>T (p.Pro604=) also in patient P4. **F.** c.1501C>T (p.Arg501Trp). **G.** c.1647G>A (p.Met549Ile). **H.** c.1839-30G>A. *Abnormal fragments on SSCP gels; "C" corresponds to control samples without the genetic change. The arrows point to the variants in the electropherograms. SSCP = single-strand conformation polymorphism.

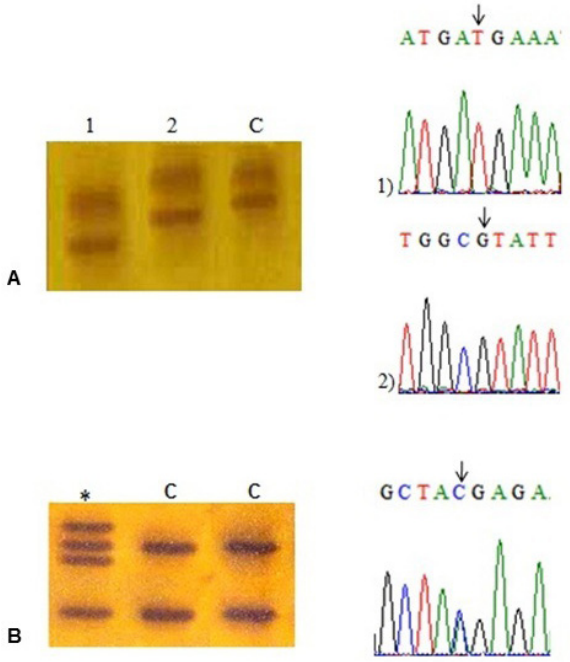

Figure 2. SSCP gels and sequencing of *CRTAP* fragments. **A.** 1: c.534C>T (p.Asp174=); 2: c.558A>G (p.Ala186=). **B.** c.1152+36C>A. *Abnormal fragments on SSCP gels; "C" corresponds to control samples without the genetic change. The arrows point to the variants in the electropherograms. SSCP = single-strand conformation polymorphism.

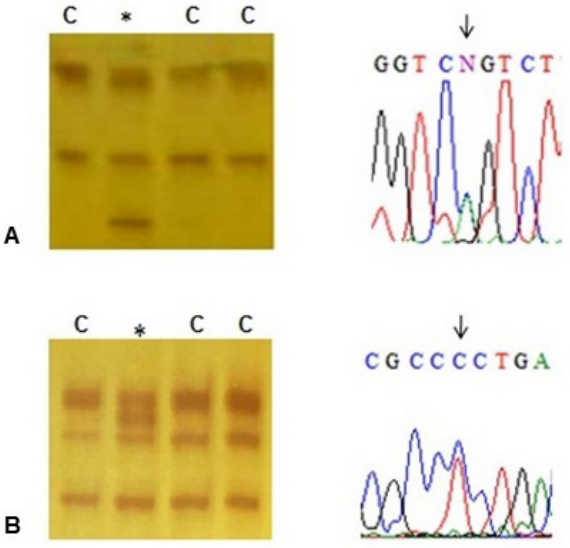

Figure 3. SSCP gels and sequencing of *PPIB* fragments. **A.** c.63C>A (p.Ser21=). **B.** c.344-27C>T. *Abnormal fragments on SSCP gels; "C" corresponds to control samples without the genetic change. The arrows point to the variants in the electropherograms. SSCP = single-strand conformation polymorphism.

LEPRE1

In this study, we detected five genetic variations in *LEPRE1* in patients with OI that were absent in control samples.

One of the patients (P1), diagnosed with OI type III, presented a homozygous splice site mutation (c.1080+1G>T) (Figure 1A). DNA of relatives was not available. P1 is son of a consanguineous couple (his parents are cousins) and is the only diagnosed case of OI in his family.

A second patient (P2), also diagnosed with OI type III, presented a heterozygous substitution (c.1087A>G) of the seventh nucleotide in exon 6, leading to an amino acid change from lysine to glutamic acid (p.Lys363Glu) (Figure 1B). P2 is the only affected patient in his family and his parents reported absence of any consanguinity.

A c.2024G>T/p.Trp675Leu heterozygous mutation, located in exon 14, was detected in a patient with OI type III (P3). The unaffected parents of P3 claimed no consanguinity. DNA from both parents and a younger sister also affected by OI type III was analyzed. The mutation was present in the mother and sister, but was absent in the patient's father (Figure 1C4).

Samples from P2 and P3 were also Sanger sequenced for all exons of the *LEPRE1* gene, but a second mutation was not identified in either patient.

Patient P4 presented both c.1720+52C>T and c.1812C>T (p.Pro604=) variations, located in intron 11 and exon 12, respectively (Figure 1D and E). P4 and his mother were both affected by OI type I. Analysis of the patient's mother's DNA revealed that she did not carry the variations detected in P4. DNA of the patient's unaffected father was not available for testing.

In addition, six different polymorphisms were detected in the *LEPRE1* gene of patients with OI and in control samples: c.1501C>T (p.Arg501Trp), c.1647G>A (p.Met549Ile), c.1839-30G>A, c.1915-20T>G, c.2055+17C>T, and c.2055+74T>A (Figure 1C, F, G, and H).

CRTAP

A c.558A>G (p.Ala186=) synonymous mutation was detected in a homozygous state in exon 2 of one patient (P5) with OI type IV (Figure 2A2). P5 is the only diagnosed patient with OI in the family and her parents declared no consanguinity. Two polymorphisms were also detected in patient and control samples: c.534C>T (p.Asp174=) and c.1152+36C>A (Figure 2A1 and C).

PPIB

An intronic mutation (c.344-27C>T) in the heterozygous state was detected in one patient with OI type I (P6) but was absent in the 100 control samples and in the patient's mother (Figure 3B). P6 is the only affected patient in his family and he is the son of a non-consanguineous couple. In addition, one polymorphism in exon 1 (c.63C>A/p.Ser21=) of patients and control samples was detected (Figure 3A).

The allelic frequencies of polymorphisms detected in patients and controls are described in Table 4. A summary of mutations identified in patients with OI and absent in 100 control samples is reported in Table 5.

Table 4. Allelic frequencies of polymorphisms detected in patients and controls.

Number	Genetic variation	Gene	Localization	Allele frequency*
1	c.2055+74T>A	*LEPRE1*	Intron 14	11.6% (29/250)
2	c.2055+17C>T	*LEPRE1*	Intron 14	8.8% (22/250)
3	c.1915-20T>G	*LEPRE1*	Intron 13	9.2% (23/250)
4	c.1839-30G>A	*LEPRE1*	Intron 12	58.4% (146/250)
5	c.1647G>A (p.Met549Ile)	*LEPRE1*	Exon 11	0.8% (2/250)
6	c.1501C>T (p.Arg501Trp)	*LEPRE1*	Exon 10	1.6% (4/250)
7	c.534C>T (p.174Asp=)	*CRTAP*	Exon 2	12.0% (30/250)
8	c.1152+36C>A	*CRTAP*	Intron 6	9.2% (23/250)
9	c.63C>A (p.Ser21=)	*PPIB*	Exon 1	5.2% (13/250)

*Allele frequency among all participants, including 25 unrelated patients (50 alleles) and 100 normal controls (200 alleles), totaling 125 Brazilian individuals (250 alleles).

Table 5. Genetic changes detected in patient samples.

	Patient	Mutation	Gene	Zygosity	Type of mutation	Predicted as disease causing	OI type	Novel or Known
1	P1	c.1080+1G>T	*LEPRE1*	Hom	Splice site	Yes	Severe	Known
2	P2	c.1087A>G (p.Lys363Glu)	*LEPRE1*	Het	Missense	No	Severe	Novel
3	P3	c.2024G>T (p.Trp675Leu)	*LEPRE1*	Het	Missense	Yes	Severe	Novel
4	P4	c.1720+52C>T	*LEPRE1*	Het	Intronic	No	Mild	Novel
5	P4	c.1812C>T (p.Pro604=)	*LEPRE1*	Het	Synonymous	No	Mild	Novel
6	P5	c.558A>G (p.Ala186=)	*CRTAP*	Hom	Synonymous*	Yes	Moderate	Novel
7	P6	c.344−27C>T	*PPIB*	Het	Intronic	No	Mild	Known

Hom = homozygous; Het = heterozygous; OI = osteogenesis imperfecta. *Predicted splice site mutation.

DISCUSSION

In this study, we analyzed 25 unrelated patients with OI and identified sixteen different genetic variations in the *LEPRE1*, *CRTAP*, and *PPIB* genes, five of which have not been previously reported. Of the total, 3 mutations were predicted as disease causing. The majority of mutations were located in *LEPRE1*, whereas the analysis of *PPIB* did not show pathogenic changes.

Among the variations detected in *LEPRE1*, we identified the homozygous splice site mutation c.1080+1G>T in a patient (P1) with a severe form of OI. This mutation, detected in one or in both alleles, had already been reported more than 50 times in different studies (Dalgleish, 1998). According to Cabral et al. (2007), who first reported the c.1080+1G>T mutation, the mutant allele had originated in West Africa and was transported to America. The mutation results in altered splicing, leading to premature translational termination and decreased levels of *LEPRE1* mRNA. Once the normal production of P3H1 is reduced, the effect on type I collagen molecules is expected to be damaging.

We detected the c.1087A>G/p.Lys363Glu variation in a heterozygous state in one patient (P2) with OI type III. This mutation had not been reported in the reference databases “Human Collagen Mutation Database 1998” (Dalgleish, 1998) and “Single Nucleotide Polymorphism Database (dbSNP)” (Kitts and Sherry, 2002), both accessed in August 2014. It is known that splice site mutations and amino acid changes might be damaging for protein formation. However, prediction programs suggest that p.Lys363Glu is a benign polymorphism in the European American population (Schwarz et al., 2014).

The c.2024G>T/p.Trp675Leu heterozygous mutation, located in exon 14, was detected in a second patient with OI type III (P3). This patient has unaffected parents and one sister also

diagnosed with OI. The patient's sister and mother, but not the father, carry the same variation. The p.Trp675Leu mutation is predicted to be disease causing (Schwarz et al., 2014). It results in a change from tryptophan, an aromatic amino acid, to leucine, a nonpolar amino acid, affecting the molecular structure of the protein. The second causative mutation was not detected in the patient, even after sequencing all exons of the *LEPRE1* gene. It is possible that the second mutation is located in a non-coding region of *LEPRE1* not analyzed in this study.

Patient P4 presented both c.1720+52C>T and c.1812C>T (p.Pro604=) variations, in intron 11 and exon 12, respectively. Both changes are absent in the patient's affected mother, suggesting that the changes are not the cause of OI in P4. This conclusion is also supported by the location of the intronic change in c.1720+52C>T and by the synonymous effect of c.1812C>T, which does not lead to an amino acid change (p.Pro604=). In addition, the family history, involving a parent and a child affected by OI, indicates an autosomal dominant pattern, which differs from the expected profile for mutations in *LEPRE1*.

Six different polymorphisms were detected in the *LEPRE1* genes of patients with OI and controls: c.2055+74T>A, c.2055+17C>T, c.1915-20T>G, c.1839-30G>A, c.1647G>A (p.Met549Ile), and c.1501C>T (p.Arg501Trp). The first four are common intronic variations not associated with OI. The c.1647G>A (p.Met549Ile) variant, despite involving an amino acid change, is described as a benign polymorphism in the African American and in the European American populations. As c.1647G>A (p.Met549Ile) was detected in both patient and control samples in the present study, we suggest that it is also a non-pathogenic polymorphism in the Brazilian population. However, the last polymorphism detected (c.1501C>T/p.Arg501Trp) is predicted to be disease causing in the African American and in the European American populations (Schwarz et al., 2014). As it was also detected in control samples in our study, we infer that the pathogenicity occurs when the mutation is in a homozygous state, rather than heterozygous as detected here.

Three genetic changes were detected in *CRTAP*. A homozygous c.558A>G (p.Ala186=) mutation was detected in one patient (P5) with OI type IV. This mutation, absent in one hundred control samples, is predicted to be disease causing as it alters a splice site (Schwarz et al., 2014). Thus, we suggest that c.558A>G (p.Ala186=) might be associated with OI in this patient.

In addition, a variant inside *CRTAP* exon 2 that does not lead to an amino acid change (c.534C>T/p.174Asp=) was detected as well. A previous study reported that this SNP might be associated with differences in bone mineralization. A positive association of the C allele and bone mineralization density in Chinese women has been established (Li et al., 2010). Although it is not probable that this variation is the cause of OI as it was also detected in unaffected control samples, we suggest that patients with OI due to another mutation might present different clinical phenotypes in the presence of the c.534C>T polymorphism.

Finally, the variant detected in intron 6 (c.1152+36C>A) of patients and control samples most likely represents a common polymorphism in *CRTAP*, not associated with OI.

In the *PPIB* gene, two genetic changes were detected. The c.344-27C>T change is a previously described heterozygous intronic variant with unknown consequences to human phenotypes (Exome Variant Server, accessed August 2014). Because this variation was detected in a non-coding region, we suggest that c.344-27C>T might be a rare genetic change not associated with the development of OI. The second *PPIB* variant is a single nucleotide substitution that does not change the amino acid (c.63C>A/p.Ser21=). In addition, its presence in unaffected control samples also suggests that it is a non-pathogenic polymorphism.

In addition to *LEPRE1*, *CRTAP*, and *PPIB*, a large number of recessive OI genes have

been described. Hence, the patients without identified mutation in this study probably carry causative mutations in other genes related to OI.

SSCP screening is described as a technique with limited efficiency for mutation detection. However, in this analysis, we improved the sensitivity of the technique by testing each sample on three distinct gels with different polyacrylamide concentrations. Furthermore, all fragments were engineered to be shorter than 400 bp because it is known that in this condition the accuracy of the SSCP technique is improved.

In conclusion, sixteen mutations and polymorphisms in *LEPRE1*, *CRTAP*, and *PPIB* were detected after analysis of 25 patients with OI. It was verified that *LEPRE1* is the gene that contains the majority of the genetic alterations (11/16), including both polymorphisms and potentially deleterious mutations. To date, there have been no other descriptions of mutations in the *LEPRE1*, *CRTAP*, and *PPIB* genes in Brazilian samples and this study provides the first information about the mutational pattern of these genes in our population. The results strengthen the proposition that *LEPRE1* should be the first gene analyzed in mutation detection studies in patients with recessive OI.

Conflicts of interest

The authors declare no conflict of interest.

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