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Examination of *OPG*, *RANK*, *RANKL* and *HIF1A* polymorphisms in temporomandibular joint ankylosis patients☆

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ABSTRACT

Purpose: To evaluate the association between polymorphisms in genes that regulate bone metabolism, such as *OPG*, *RANK*, *RANKL*, and *HIF1A*, in patients with temporomandibular joint (TMJ) ankylosis.
Methods: The sample consisted of 181 individuals, the study included 17 individuals with TMJ ankylosis and 164 controls. DNA was extracted from buccal epithelial cells. The genotyping of genetic polymorphisms in *OPG* (*rs2073618*), *RANK* (*rs3826620*), *RANKL* (*rs9594738*), and *HIF1A* (*rs2301113* and *rs2057482*) was performed by real-time PCR using TaqMan™ technology (Applied Biosystems). The data were subjected to statistical analysis with a level of significance of 0.05.
Results: The *OPG* (*rs2073618*) polymorphism was associated with TMJ ankylosis, both in the additive model and in the dominant model ($p < 0.05$). In the additive model, when the individuals carried the CC genotype, they presented as 10.80 times more likely to develop the condition ($p = 0.03$). In the dominant model, individuals that carried at least one C allele were 5.76 times more likely to have TMJ ankylosis, than those with the G allele ($p = 0.01$).
Conclusion: The polymorphism *rs2073618* of *OPG* is a possible marker that is associated with the risk of manifestation of TMJ ankylosis.

1. Introduction

Temporomandibular joint (TMJ) ankylosis is an uncommon and complex disease that has negative effects on masticatory, phonatory, and respiratory functions (Manganello-Souza and Mariani, 2003; Maki and Al-Assaf, 2008). TMJ ankylosis is always aggressive and varies according to the degree of involvement of the structures (Ma et al., 2015). The etiology of TMJ ankylosis is multifactorial and has been associated with certain conditions, such as facial trauma, poorly treated otitis, and other unknown factors (Posnick and Goldstein, 1993). Additionally, genetic and molecular bone factors may play an important role in the triggering of this dysfunction (Gu et al., 2008; Huang et al., 2011; Li et al., 2014; Yan et al., 2014); however, the genes involved in this condition are largely unknown.

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The structural integrity of mature bone is preserved through the process of bone remodeling, in which bone formation and bone resorption are simultaneously balanced at the same place (Boughner et al., 2007; Yang, 2009). Osteoblasts and osteoclasts participate in the process of bone remodeling and are regulated by many molecular factors (Shao et al., 2015). Osteoprotegerin (*OPG*), receptor activator of nuclear factor-kB (*RANK*), and the *RANK* ligand (*RANKL*) system are markers of bone metabolism. *RANKL* and *OPG* are members of the tumor necrosis factor (TNF) and TNF receptor (*TNFr*) superfamilies, respectively, and they bind to RANK. This system was also identified as important for bone homeostasis through the regulation of osteoclasts (Lacey et al., 1998).

Hypoxia-inducible factors (HIFs) are transcription factors that respond to alterations in the available oxygen in the cellular environment. Hypoxia-inducible factor 1-alpha (*HIF1A*) is a master regulator of cellular and developmental oxygen homeostasis by activating the transcription of many genes (Semenza, 2000). *HIF1A* of osteoblasts and osteoclasts has received increased attention (Shao et al., 2015; Martinez et al., 2010; Hulley et al., 2017). Some studies have demonstrated that mesenchyme-derived marrow stroma cells, osteoblasts, and chondrocytes are oxygen sensing cells that express *HIF1A* and regulate its functional adaptation (Wan et al., 2010; Tamama et al., 2011). *HIF1A* could mediate the transition from angiogenesis to osteogenesis (Wang et al., 2007), and likely suppresses the number and activity of osteoclasts (Shao et al., 2015). It has been demonstrated that hypoxia upregulates *RANK* and *RANKL* expression and increases *RANKL*-induced cell migration via the *HIF1A* pathway (Tang et al., 2011). Additionally, a high level of *HIF1A* was demonstrated to stimulate *OPG* expression and through this signaling pathway, downregulate the activity of osteoclasts (Shao et al., 2015). TMJ ankylosis is a complex alteration involving some etiological factors, however, the specific role of the genes in its etiology is still largely unknown. Therefore, this study aimed to evaluate the association between TMJ ankylosis and genetic polymorphisms in *OPG, RANK*, *RANKL* and *HIF1A*.

2. Methods

2.1. Subjects

The Local Ethics Committee approved this study (#58068716.4.0000.0093), and it complied with the tenets of the Declaration of Helsinki for studies involving human subjects. Informed consent was obtained from all participating individuals or parents/legal guardians. Data collection was performed between August 2016 and March 2017.

This study included subjects of both genders, without age restrictions, who were diagnosed with TMJ ankylosis that may or may not have undergone surgical procedures to treat the pathology. The control group was comprised of patients without TMJ ankyloses, who were seeking dental treatment.

The epidemiological data were collected from medical and dental records. DNA was obtained from buccal mucosa epithelial cells by a 5 mL rinse with a 3% glucose solution for 2 min and scraping of the buccal mucosa with a sterilized wooden spatula (Trevilatto and Line, 2000).

2.2. PCR and Genotyping

The characteristics of the studied genetic polymorphisms are presented in Table 1.

The genetic polymorphisms in *OPG* (*rs2073618)*, *RANK* (*rs3826620)*, *RANKL* (rs9594738), and *HIF1A* (rs2301113 and rs2057482) were genotyped using real-time PCR (StepOnePlus™ Real-time PCR System) with the TaqMan™ assay (Applied Biosystems, Foster City, CA, USA) in a StepOnePlus Real-Time PCR System (Applied Biosystems). The real-time PCR reactions were performed in a total volume of 3 μl (4 ng DNA/reaction, 1.5 μl Taqman PCR master mix, 0.075 SNP assay; Applied Biosystems, Foster City, CA). The thermal cycling was carried out by starting with a hold cycle of 95 °C for 10 min, followed by 40 amplification cycles of 92 °C for 15 s and 60 °C for 1 min. Two negative controls were used per plate. Additionally, 10% random samples were chosen for repeat analysis, the outcome was 100% consistent.

2.3. Statistical Analysis

The association between the genotypes, under additive, recessive, and dominant models between the groups (TMJ ankylosis and control), was assessed using the logistic univariate regression model or Fisher's exact test, when one of the genotypes was equal to zero. The odds ratios (OR) were also calculated. Values of $p < 0.05$ indicated statistical significance. The data were analyzed using the SPSS (*Statistical Package for Social Science, v.24, IBM, USA*).

3. Results

The sample consisted of 181 individuals: 17 individuals with TMJ ankylosis and 167 controls. The etiologies of the TMJ ankylosis group included 7 individuals with prior surgery in TMJ (41.18%), 4 with an automobile accident history (23.53%), 3 with otitis history (17.64%), 1 with forceps delivery trauma (5.89%), and 2 with unknown causes (11.76%). The patients who were classified as individuals with prior surgery of the TMJ corresponding to performed surgical intervention in the TMJ such as disk replacement, for example. Eight of the individuals were male (47.05 %), and 9 were female (52.95%). The age of the TMJ ankylosis group ranged from 6 to 57.

An *OPG* gene polymorphism was associated with TMJ ankylosis, both in the additive model and in the dominant model. In the additive model, when the individual carried the CC genotype, there was a 10.80 times greater chance of presenting with TMJ ankylosis ($p = 0.03$). In the dominant model, individuals caring at least one C allele were 5.76 times more likely to have TMJ ankylosis than the individuals with the G allele ($p = 0.01$). The other genes were not associated with TMJ ankylosis ($p > 0.05$) (Table 2).

Table 1
Candidate studied genes and polymorphisms.

Gene	Position	Reference sequence	Type of alteration[♀]	Base Change (Context sequence) *	Global MAF*	Assay ID[⊥]
OPG	8q24.12	*rs2073618*	Missense[a]	CAA **[C/G]**TTG	0.333/1669	Hs00297304_CE
RANK	18q21.33	*rs3826620*	intron variant	CAC **[A/G/T]**CTG	0.349/1748	C__25474631_10
RANKL	13q14.11	*rs9594738*	intron variant	CTA **[C/T]**GAA	0.284/1425	C__30459982_10
HIF1A	14q23.2	*rs2301113*	intron variant	CCA [**A/C**]AGG	0.470/2354	C__15755917_10
HIF1A	14q23.2	*rs2057482*	intron variant,	TCA **[C/T]**TAC	0.242/1214	C___8549084_20

Note: Bold form indicates ancestral allele; MAF means minor allele frequency. Data obtained from databases: *http://www.ncbi.nlm.nih.gov/snp/; ♀http://genome.ucsc.edu/; ⊥http://www.thermofisher.com/.

[a] (Lys > Asn).

Table 2
Genotype and allele distributions between control and case group.

	Gene	Genotype	Case	Control	P value	Ods Ratio (CI)
OPG rs2073618	Additive	CC	1 (11.1%)	54 (32.9%)	0.03	10.80 (1.13–102.52)
		CG	4 (44.4%)	90 (54.9%)	0.04	4.50 (1.03–19.53)
		GG	4 (44.4%)	20 (12.2%)	reference	–
	Dominant	CC/CG	5 (55.6%)	144 (87.8%)	0.01	5.76 (1.42–23.25)
		GG	4 (44.4%)	20 (12.2%)	reference	–
	Recessive	CG/GG	8 (88.9%)	110 (67.1%)	0.20	0.25 (0.03–2.08)
		CC	1 (11.1%)	54 (32.9%)	reference	–
RANK rs3826620	Additive	GG	2 (15.4%)	69 (42.3%)	0.50	2.3 (0.19–27.0)
		GT	10 (76.9%)	79 (48.5%)	0.55	0.52 (0.06–4.42)
		TT	1 (7.7%)	15 (9.2%)	Reference	–
	Dominant	GG/GT	13 (100.0%)	148 (90.8%)	0.60[a]	–
		TT	0 (0.0%)	15 (9.2%)		
	Recessive	GT/TT	10 (76.9%)	94 (57.7%)	0.18	0.40 (0.10–1.54)
		GG	3 (23.1%)	69 (42.3%)	reference	–
RANKL rs9594738	Additive	CC	8 (61.5%)	65 (39.9%)	0.45	0.54 (0.10–2.70)
		CT	3 (23.1%)	68 (41.7%)	0.66	1.51 (0.24–9.51)
		TT	2 (15.4%)	30 (18.4%)	reference	–
	Dominant	CC/CT	11 (84.6%)	133 (81.6%)	0.78	0.80 (0.17–3.82)
		TT	2 (15.4%)	30 (18.4%)	reference	–
	Recessive	CT/TT	5 (38.5%)	98 (60.1%)	0.13	2.41 (0.75–7.69)
		CC	8 (61.5%)	65 (39.9%)	reference	–
HIF rs2301113	Additive	AA	7 (50.0%)	56 (34.1)	0.22	0.26 (0.03–2.27)
		AC	6 (42.9%)	78 (47.6%)	0.44	0.44 (0.05–3.75)
		CC	1 (7.1%)	30 (18.3%)	reference	–
	Dominant	AA/AC	13 (92.9%)	134 (81.7%)	0.31	0.34 (0.04–2.72)
		CC	1 (7.1%)	30 (18.3%)	reference	–
	Recessive	AC/CC	7 (50.0%)	108 (65.9%)	0.24	1.92 (0.64–5.77)
		AA	7 (50.0%)	56 (34.1)	reference	–
HIF rs2057482	Additive	CC	7 (58.3%)	76 (63.3%)	0.47[a]	–
		CT	5 (41.7%)	35 (29.2%)		
		TT	0 (0.0%)	9 (7.5%)		
	Dominant	CC/CT	12 (100.0%)	111 (92.5%)	1.00[a]	–
		TT	0 (0.0%)	9 (7.5%)		
	Recessive	CT/TT	5 (41.7%)	44 (36.7%)	0.77	1.19 (0.35–3.97)
		CC	7 (58.3%)	75 (62.5%)	reference	–

Note: Logistic univariate regression model, with significance level of 0.05.
CI: Confidence Interval.
[a] *Fisher's Exact* Test was used.

4. Discussion

The reason for the occurrence of traumatic TMJ ankylosis is still a mystery, partly because of the low incidence of ankylosis after TMJ trauma, which fails to permit the identification of a main external factor that is involved in its etiology (Yan et al., 2014; Laskin, 1978). Therefore, this highlights the hypothesis that TMJ ankylosis has a strong genetic background involved in its etiology. In fact, in the past twenty years, studies with animal models and human samples have demonstrated that some genes may play a key role in the etiology of TMJ ankylosis through different pathways. A study by Gu et al. (2008) with an animal model showed that shox2-deficient mice developed TMJ fibrous ankylosis (Gu et al., 2008). Another study, performed with both an animal model and human samples, identified TMJ ankylosis in *ank* mutant mice. For the human samples, the authors found that a genetic polymorphism in *ANKHOR* was associated with TMJ ankylosis and could be a genetic marker that is associated with this condition (Huang et al., 2011).

Our present study added some novel information regarding the etiological factors of TMJ ankylosis, as it indicates that the genetic polymorphism rs2073618 of *OPG* could be a marker that is associated with this dysfunction. Vistoropsky et al. (2007) evaluated the association between *ANKH* polymorphisms and variations in the OPG level, and interestingly found a highly significant association between the OPG plasma levels and intronic genetic polymorphisms in *ANKH* (Vistoropsky et al., 2007). Many researchers have demonstrated the critical role that the *RANK*, *RANKL*, and *OPG* system plays in bone biology and their associations with many diseases and conditions (Walsh and Choi, 2014). *OPG* and *RANKL* are essential regulators that couple bone formation and resorption. *OPG* acts as a decoy receptor and blocks the effect of *RANKL*, which then increases the production, activity, and survival of osteoclasts (Sakakura et al., 2005). Therefore, polymorphisms that increase the activity of *OPG* would be expected to have an impact on bones, due to increased resorption (Roshandel et al., 2011). In our study, the polymorphism rs2073618 of *OPG* was demonstrated to be a biomarker for disease occurrence, and it was associated with a higher chance of having TMJ ankylosis. Conditions with genetic alterations in *OPG*, such as juvenile Paget's disease (Naot et al., 2014) and idiopathic hyperphosphatasia (Chong et al., 2003), reinforce the role of *OPG* in some pathological conditions.

A previous study evaluated bone mineral density and concluded that the *Asn–Asn* genotype of the rs2073618 polymorphism of *OPG* was associated with lower serum *OPG* levels, compared to the *Lys–Lys* genotype (Zhao et al., 2005). In our study, the *Asn–Asn* polymorphism was the variant associated with TMJ ankylosis. Additionally, another study evaluating this polymorphism demonstrated that this genetic variation was associated with higher bone turnover markers (Roshandel et al., 2011).

HIF1A is expressed in most human tissues. It appears that *HIF1A* plays a general role by signaling the existence of hypoxia to the transcriptional machinery in the nucleus of all cells (Semenza, 2000). Here, the two evaluated genetic polymorphisms were not associated with TMJ ankylosis; neither interacted with the studied genetic polymorphisms of the *RANK, RANKL,* and *OPG* system. In fact, a further study showed that *OPG* was upregulated in situations with a high level of *HIF1A*, while *RANKL* was unaffected. The authors

hypothesized that *HIF1A* may directly bind to the promoter site of *OPG* and stimulate its transcription (Shao et al., 2015). It is possible that other polymorphisms in *HIF1A* could be involved in the etiology of TMJ ankylosis.

It is important to emphasize the control group selection approach used here. It is known that in the evaluation of a rare disease, as is the case for TMJ ankylosis, the number of affected individuals included in the research is small. In the literature from 2018, studies evaluating the aspects and treatment approaches for TMJ ankylosis included 6 (Qiao et al., 2018), 10 (Yang et al., 2018), 15 (Fariña et al., 2018), 18 (Jiang et al., 2018), 35 (Singh et al., 2018), and 40 (Zhang et al., 2018) individuals with TMJ ankylosis. Therefore, increasing the number of individuals in the control group was a strategy that allowed us to increase the statistical power of the study. In addition, the control group design was performed to be representative of the population, because it is unknown who is or is not exposed to a predisposing factor, such as trauma or otitis in childhood, in order to select a control group that would have been exposed to environmental factors and did not develop TMJ ankylosis. However, this approach has some obvious limitations and future studies should be performed using controls more closely matched with the cases, such as facial trauma patients who develop TMJ ankyloses versus those with similar injuries that did not develop TMJ ankylosis or patients with history of otitis who develop TMJ ankyloses versus those that did not. Future studies should include a matched and large control group in order to replicate and validate our results.

Briefly, the polymorphism rs2073618 of *OPG* was presented as a potential marker that is associated with the risk of manifestation of TMJ ankylosis. Therefore, further studies should be performed in order to identify the genetic etiology of TMJ ankylosis, as well as to evaluate the interaction between *ANKHOR* polymorphisms and OPG. Knowledge of the genes involved in the etiology of TMJ ankylosis will impact the future of prevention and treatment for this condition.

5. Conclusion

The genetic polymorphism rs2073618 (Lys > Asn) in OPG was associated with the risk of manifestation of TMJ ankylosis and is a possible marker for this condition. Further studies are necessary to confirm this association. The evaluation of OPG and TMJ ankylosis should also be expanded to functional genic expression analysis in ankylosing bone and OPG sequencing. In the future, it will be possible to identify individuals with a genetic predisposition to TMJ ankylosis.

Conflict of interest statement

The authors confirm that there are no known conflicts of interest associated with this publication.

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